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13. ABSTRACT ***(Maximum 200 Words)***
Heregulin is a glycoprotein ligand that interacts with the receptor tyrosine kinases, ErbB3 and ErbB4. This interaction induces a dimerization with and subsequent activation of the Neu/ErbB2 protein, which it turn initiates cellular signaling cascades. The signaling pathways that function as a consequence of Neu/ErbB2 activation are of great interest as the overexpression of Neu/ErbB2 is found in up to 40% of breast cancers and correlates with a poor prognosis for women with breast cancer. Mapping the molecular determinants of the heregulin/ErbB2 signaling pathway will be important in determining viable cellular targets for therapeutic intervention. To this end, we have identified the nuclear cap-binding complex (CBC) as a novel target for heregulin signaling. As the CBC plays an important role in the regulation of gene expression at the level of RNA processing, it is likely that heregulin can affect cell growth by altering the expression of genes important for mitogenesis. Indeed, we have shown heregulin to promote mRNA splicing, presumably through increasing the affinity of the CBC for capped RNAs. Additionally, we have determined some of the fundamental players in heregulin signaling to the CBC, including Cdc42, FRAP, and S6 kinase. Transforming alleles of Cdc42, like heregulin, can increase mRNA splicing, while a specific Cdc42 mutant which can no longer transform cells is no longer able to promote RNA splicing. Together these data identify a specific heregulin-Neu/ErbB2 pathway, and suggest a CBC-directed mechanism by which the activation of this pathway affects cell growth by altering post-transcriptional gene expression.

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INTRODUCTION

Members of the epidermal growth factor (EGF) receptor subfamily of receptor tyrosine kinases, including Neu/ErbB2, ErbB3 and ErbB4 have been implicated in the development of different cancers, including breast cancer [1-6]. Heregulin is a physiological ligand for this class of receptors [7-9] and interacts directly with either ErbB3 or ErbB4 to induce a dimerization of these receptors with Neu/Erb2, and subsequent signal propagation. The potency of this signal in cellular function is underscored by the observation that the overexpression of Neu/ErbB2 is correlated with a poor prognosis for women diagnosed with breast cancer [2, 4]. An understanding of the molecular events which arise as a result of the heregulin stimulus is fundamental to our understanding of how cell growth is controlled, or loses control, as a result of the expression of ErbB receptors. The purpose of the original proposal on which this work has been based, was to uncover the molecular mechanisms of heregulin signaling, specifically by elucidating the identity of a nuclear protein which responded to the heregulin treatment of cells with an increased incorporation of [α^{32}P]GTP in a photoaffinity labeling assay. Over the course of the funding period, we identified this heregulin-responsive, nuclear protein as the 20 kDa subunit of the nuclear cap-binding complex (CBC) [10]. This protein, CBP20, along with its 80 kDa binding partner, CBP80, recognizes and binds to the ^{7}methylguanosine cap-structure on RNAs transcribed by RNA polymerase II, including mRNAs and U snRNAs [11]. In this capacity, the CBC has been found to enhance RNA splicing, export, and 3'end formation [11-16]. A suggestion from this finding would be that one way in which heregulin signaling could affect cells would be to influence gene expression at the level of RNA processing. Indeed, this is born out by our observation that the heregulin treatment of cells activates the splicing machinery [10]. Subsequent to our identification of the CBC as a nuclear target for heregulin, we have sought to piece together the signal transduction pathway that begins with an interaction between heregulin and its cognate receptors, and terminates in an activation of the CBC to bind capped RNAs. To this end, we have identified the low molecular weight GTP-binding protein, Cdc42 and the pp^{70} S6 kinase (S6K) as major determinates in the signaling pathway leading to the CBC, and have evidence to support a direct activation of the CBC by S6K [17]. Interestingly, activated alleles of Cdc42 are capable of transforming NIH 3T3 cells [18] as well as activating the CBC and causing an increase in mRNA splicing [10, 17]. Together, these observations support a role for this signal transduction pathway in the mitogenic affects induced by heregulin.

BODY

The premise of our original proposal was that understanding the basic molecular mechanisms of heregulin signaling would be fundamental to an understanding, and eventual treatment of breast cancers where an overexpression of Neu/ErbB2 is observed. At the core of our proposal was an 18 kDa nuclear protein (originally designated p18) which we had observed to be heregulin-responsive. Additionally, this protein responded to other growth factors, G1/S arrest, and conditions of cellular stress, pointing to the likelihood that this nuclear protein was involved in some aspect of cell growth control. Therefore, by accomplishing a set of three Tasks, we proposed to characterize a heregulin-stimulated signal transduction pathway to the nucleus, which resulted in the activation of p18. Two manuscripts and one review [**Appendices I-III**] have resulted from this endeavor. These manuscripts will be used for a detailed description of the work that has been completed in regard to the Tasks in this final report. The results from the Tasks will briefly be described below according to the chronology in which they were accomplished.

Task 2. Purification and molecular cloning of p18.

The second Task was the first accomplished and was reported in both the 1998 progress report and in **Appendix I**. As stated above, we were interested in elucidating the molecular identity of a nuclear protein which responded to heregulin treatment with an enhanced incorporation of [α^{32}P]GTP as assayed using a photoaffinity labeling technique. To this end, we attempted to purify sufficient quantities of this protein to obtain microsequencing information. Bovine retina were used as a protein purification source as this tissue appeared to be particularly enriched in p18 activity. This activity was separated away from other retinal proteins by a series of classical chromatography procedures (see **Appendix I** for experimental detail), and while we were effective in enriching p18 activity, as well as detecting an 80 kDa species which appeared to copurify with p18, we had difficulty isolating the desired quantities of the protein for microsequencing analysis. Simultaneously, we employed a computer-based approach to search genomic data-bases for putative GTP-binding proteins that fit the characteristics of p18. Using this approach, we identified a nuclear cap-binding protein, CBP20, which shared similarities to p18 such as its molecular weight and cellular localization, its association with an 80 kDa protein (CBP80) and its ability to be crosslinked to substrate by UV irradiation. Using antibodies against CBP20 and CBP80 (a generous gift of Dr. Iain Mattaj, Heidelberg, Germany), we confirmed by Western blotting that these proteins were highly abundant in our partially purified p18 preparations, suggesting the possibility that p18 was indeed CBP20. CBP20 and CBP80 were cloned and protein expression systems were established for *E. coli, S. frugiperda*, and mammalian protein expression (see **Appendix I** and **Appendix II**). Once establishing these systems, we were able to conclude from a series of *in vivo* and *in vitro* experiments that p18 and CBP20 were identical (**Appendix I,** Figures 1, 2, and 3). Thus, as a direct result of Task 2, we identified the CBC as a nuclear target for heregulin, and developed recombinant protein expression systems that would facilitate Task 1.

Task 1. Development of cell systems containing active and dominant-negative mutants of Cdc42 and Rac for examining the involvement of these GTP-binding proteins and related signaling molecules in the activation of p18.

Once p18 was conclusively identified as the 20 kDa subunit of the CBC, we next endeavored to understand the signal transduction events which culminated in an activation of the CBC to bind capped RNAs. This work was the focus of the progress

report for 1999, and has been continued through to the present. Details from these studies can be found in **Appendix I** and **Appendix II**.

By transiently transfecting constitutively active forms of Cdc42 and Ras into HeLa cells, we were able to bypass the requirement for heregulin in the activation of the CBC (**Appendix I,** Figure 5C), thus suggesting that these GTP-binding proteins may transduce the heregulin signal to the nucleus. Ideally, we would like to confirm this by blocking the heregulin-stimulated activation of the CBC with dominant negative forms of these GTP-binding proteins. Unfortunately, we have not been able to accomplish such a block, which we believe may be due to an insufficient expression of the dominant negative proteins. Nonetheless, parallels exist between the effects of heregulin and constitutively active Cdc42 on the CBC in our system, such that we believe it plausible that the heregulin effects on the CBC are being mediated by Cdc42. Specifically, both heregulin and Cdc42 can activate the CBC (**Appendix I**), both heregulin and Cdc42 can stimulate cap-dependent RNA splicing (**Appendix I,** Figure 5 and **Appendix II**, Figure 1), and both heregulin and Cdc42 result in a phosphorylation of CBP80 *in vivo*. Thus, we have continued to develop the participants of the Cdc42 pathway leading to the CBC and find that the activation of the CBC downstream of Cdc42 is dependent on FRAP and S6 kinase. Rapamycin, a small molecule inhibitor of FRAP (and subsequently S6 kinase), blocks the ability of Cdc42 to activate the CBC (**Appendix II**, Figure 2A). Additionally, the transient expression of S6 kinase when expressed with one of its upstream activators (a constitutively active form of PI3 kinase) leads to an activation of the CBC (**Appendix II**, Figure 2B).

Not only do we believe that S6 kinase participates in signaling to the CBC, but we also suspect that it might signal to the CBC by a direct phosphorylation of CBP80. As stated above, we have observed that the growth factor treatment of cells results in a phosphorylation of CBP80 *in vivo*. The reasons we suspect that this phosphorylation may be due to S6 kinase are that (1) the *in vivo* phosphorylation of CBP80 can be blocked by rapamycin (**Appendix II**, Figure 4A and 4B), (2) CBP80 can serve as a substrate for S6 kinase *in vitro* (**Appendix II**, Figure 3), and (3) the site on CBP80 which is phosphorylated by S6 kinase *in vitro*, is also phosphorylated *in vivo* (**Appendix II**, Figure 4D).

Thus, our current understanding of the signal transduction pathway which leads to CBC activation in as follows. The signal is initiated by the interaction of heregulin with its receptors. This in turn leads to the activation of Cdc42, FRAP and then S6 kinase. Once S6 kinase is activated, it can phosphorylate CBP80, which in turn may result in either a direct activation of the CBC, or an enhancement of a CBC-directed cellular function such as RNA splicing. (Let it be noted that we have still to elucidate the series of signaling events which transpire between the activation of the receptor and the activation of Cdc42 in order to complete the steps in this pathway.) It is the consequence of the CBC binding to capped RNAs which is presumably having an effect on cell growth. While a constitutively active form of Cdc42 (F28L Cdc42) causes cellular transformation [18], CBC activation (**Appendix I**, Figure 5C) and cap-dependent RNA splicing (**Appendix II**, Figure 1A), an effector loop Cdc42 mutant (F28L, C37A) can block both RNA splicing and cellular transformation (**Appendix II**, Figure 1B). It will be the focus of future research to determine whether there exists a causal relationship between anomalous CBC-dependent gene expression and cellular transformation. In particular, it will be of great interest to determine whether there exists CBC mutants (such as phosphorylation defective CBC) which can reverse Cdc42 and/or Neu/ErbB2 mediated transformation.

Task 3. Biochemical studies of the regulation of p18.

This Task was based on an early observation that the loss of RCC1 (the guanine nucleotide for the nuclear GTP-binding protein, Ran) expression in mammalian cells

caused an increase in p18 GTP-binding activity. It was originally proposed that RCC1 might function as a guanine nucleotide dissociation inhibitor for the putative nuclear GTP-binding protein, p18. Upon identifying p18 as CBP20, we looked for an association between RCC1 and CBP20 by immunoprecipitation, and none was found. Based on reports in the literature, the Ran GTP-binding protein (which is activated by RCC1) is able to indirectly influence cap-binding by the CBC by influencing interactions between the importins and the CBC [19], thus explaining our early observation. As stated in the progress reports from 1998 and 1999, as no interaction exists between RCC1 and the CBC, this Task was no longer viable in its originally conceived form.

KEY RESEARCH ACCOMPLISHMENTS

- The CBC, a protein complex involved in post-translational gene expression, has been identified as a novel endpoint for heregulin-stimulated signal transduction pathways.
- Heregulin can stimulate the splicing machinery in quiescent cells.
- The signal transduction pathway to the CBC utilizes the GTP-binding protein Cdc42, FRAP, and S6 kinase.
- CBP80 is subject to a rapamycin-sensitive phosphorylation in response to growth factors, and this phosphorylation has been mapped to two sites in the amino terminus of CBP80.
- The S6 kinase can phosphorylate CBP80 *in vitro* at a site that is phosphorylated *in vivo*, suggesting the possibility that S6 kinase functions as a CBP80 kinase *in vivo*.
- Cdc42 can stimulate cap-dependent RNA splicing, and a mutation in Cdc42 that disrupts cellular transformation also disrupts cap-dependent RNA splicing.

REPORTABLE OUTCOMES

- Two publications on this work appear in the Journal of Biological Chemistry, and a review based on this work appears in Biological Chemistry (see **Appendices I, II and III**).
- The primary investigator received her Ph.D. from Cornell University in May, 1999.
- The primary investigator applied for and received offers for postdoctoral positions in the following laboratories:
  - Dr. Gideon Dreyfuss, University of Pennsylvania School of Medicine
  - Dr. John Lis, Cornell University
  - Dr. Rey Chen, Cornell University
  - Dr. Richard Cerione, Cornell University

  And is currently continuing her work as a postdoctoral associate in the lab of Dr. Richard Cerione.

CONCLUSIONS

The overexpression of members of the ErbB subfamily of receptor tyrosine kinases has been determined to be a contributing cause in the occurrence of many breast cancers, with up to 40% of breast cancers demonstrating an overexpression of Neu/ErbB2

[1-6]. The glycoprotein heregulin is the physiological ligand for these receptors [7-9] which, in binding to ErbB3 or ErbB4, will induce a dimerization with and subsequent activation of Neu/ErbB2 [20]. The activation of Neu/ErbB2 (which is hallmarked by its phosphorylation) serves to initiate signal transduction pathways that impact upon different cellular functions. Most important to the current topic is the positive effect that this signaling has on mitogenesis, as the overexpression of Neu/ErbB2 can result in receptor activation in the absence of ligand. Because the deregulation of signaling events caused by the overexpression of Neu/ErbB2 is associated with the development of breast cancer, it has been our aim to understand how heregulin signaling occurs in normal cells and what impact these signals have on the biological operations of the cell. We believe that this understanding would facilitate the identification of molecular points of intervention which could serve as therapeutic targets in the attempt to reverse the aberrant effects of Neu/ErbB2 overexpression.

Therefore, we sought to delineate a heregulin signaling pathway to the nucleus. As we had identified a nuclear activity that appeared to be heregulin-sensitive, we made this protein the focus of our original proposal. By revealing the molecular identity of this heregulin-sensitive activity, we hoped to accomplish two major goals. Firstly, we hypothesized that an understanding of the role that this activity plays in the cell nucleus would shed light on how heregulin affects biological function. Secondly, we could use this activity as a signaling endpoint that would allow us to map the protein intermediates which exist between the receptors and this nuclear protein. In both these regards, we have made considerable advancements in understanding at least one aspect of heregulin signaling. The heregulin-sensitive nuclear target was identified as the nuclear cap-binding complex [10], a protein complex known to play a key role in post-transcriptional gene regulation at the levels of RNA splicing, export and 3' end formation [11, 15, 16]. This suggests that heregulin can affect cell growth by manipulating gene expression at the level of the CBC. Indeed we have determine that the heregulin treatment of cells results in an activation of the splicing machinery [10]. Whether the positive effect of heregulin on CBC cap-binding results in a general increase in RNA processing, or an alteration in the profile of certain mRNA transcripts remains to be determined.

Using the CBC as a readout for ErbB2 activation upon treatment with heregulin, we have made inroads into dissecting a heregulin signaling pathway to the nucleus. We believe that the activation of ErbB2 can result in an activation of the low molecular weight GTP-binding protein, Cdc42. Activated alleles of Cdc42 have been shown to cause cellular transformation [18], reinforcing the notion that such a pathway when activated downstream of Neu/ErbB2, could contribute to the mitogenicity of Neu/ErbB2 overexpression. Cdc42 appears to be activating the S6 kinase via another signaling intermediate, FRAP. S6 kinase, in turn, can both phosphorylate the CBC within the amino terminus of the CBP80 subunit, and activate the CBC to bind to capped RNAs [17]. Again, this series of events appears to culminate in a change in gene expression, at least through a quantitative difference in general mRNA splicing. We have found that the increase in Cdc42 signaling can result in an increase in RNA splicing under transforming conditions, and a block in Cdc42-induced transformation (at least via one specific effector loop mutant) correlates with a loss of Cdc42-stimulated RNA splicing [17]. Thus, we think it likely that positive cell growth effects can be directly linked to CBC activation downstream of heregulin and Cdc42. It will be of great interest to determine whether we can attenuate these effects by introducing into cells cap analogs that would competitively inhibit the ability of the CBC to bind native capped RNAs and facilitate their further processing. If cap analogs were to reverse the effects of Cdc42 overactivation and/or Neu/ErbB overexpression, then the CBC would be an interesting target for therapeutic intervention, and a search for small molecule inhibitors to this target should be pursued.

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The Nuclear Cap-binding Complex Is a Novel Target of Growth Factor Receptor-coupled Signal Transduction*

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In an attempt to further understand how nuclear events (such as gene expression, nuclear import/export, and cell cycle checkpoint control) might be subject to regulation by extracellular stimuli, we sought to identify nuclear activities under growth factor control. Using a sensitive photoaffinity labeling assay that measured [α-^{32}P]GTP incorporation into nuclear proteins, we identified the 20-kDa subunit of the nuclear cap-binding complex (CBC) as a protein whose binding activity is greatly enhanced by the extracellular stimulation of serum-arrested cells. The CBC represents a 20- and 80-kDa heterodimer (the subunits independently referred to as CBP20 and CBP80, respectively) that binds the 7-methylguanosine cap on RNAs transcribed by RNA polymerase II. This binding facilitates precursor messenger RNA splicing and export. We have demonstrated that the [α-^{32}P]GTP incorporation into CBP20 was correlated with an increased ability of the CBC to bind capped RNA and have used the [α-^{32}P]GTP photoaffinity assay to characterize the activation of the CBC in response to growth factors. We show that the CBC is activated by heregulin in HeLa cells and by nerve growth factor in PC12 cells as well as during the G_1/S phase of the cell cycle and when cells are stressed with UV irradiation. Additionally, we show that cap-dependent splicing of precursor mRNA, a functional outcome of CBC activation, can be catalyzed by growth factor addition to serum-arrested cells. Taken together, these data identify the CBC as a nuclear target for growth factor-coupled signal transduction and suggest novel mechanisms by which growth factors can influence gene expression and cell growth.

Growth factor binding to cell-surface receptors can initiate signals that are propagated through the cell by a cascade of protein-protein interactions, ultimately to impact upon specific cellular functions and regulate cell growth. The activities of signaling molecules must be tightly regulated to maintain the integrity of cellular communication, as loss of regulation in these processes can give rise to defects in cell growth and metabolism that may lead to human disease. Given the importance of signaling processes in cell growth, a great deal of effort has gone into the elucidation of proteins participating in signaling pathways that start at the level of receptor activation and culminate in the stimulation of a nuclear activity. Multiple cascades have now been identified that result in the activation of different nuclear mitogen-activated protein kinases, including the extracellular receptor-activated kinases and the stress-responsive c-Jun N-terminal kinase/stress-activated protein kinase and p38 (1, 2). Extracellular receptor-activated kinase activation is the outcome of mitogen-stimulated Ras signaling, whereas c-Jun N-terminal kinase/stress-activated protein kinase and p38 activities are often stimulated by pathways involving the Cdc42 and Rac GTP-binding proteins (2–6). Although these different signaling pathways were originally thought to be independently regulated, later work showed that cross-talk between the individual mitogen-activated protein kinase pathways exists. A common functional outcome of the activation of these signaling pathways is a translocation of the activated mitogen-activated protein kinase to the nucleus and subsequent activation of specific transcription factors and gene expression (2–6).

How other nuclear functions might be influenced in response to extracellular stimulation is less clear. However, it is attractive to envision how critical nuclear activities such as RNA metabolism and export, nuclear protein import, and cell cycle control might be subject to regulation as downstream targets of extracellular stimuli. With this in mind, we set out to identify novel nuclear activities that were growth factor-responsive. Using a photoaffinity labeling approach, we identified the nuclear cap-binding complex (CBC)[1] as such an activity based on the enhanced ability of its ~20-kDa subunit (CBP20) to undergo a photocatalyzed incorporation of [α-^{32}P]GTP in response to extracellular stimulation. The CBP20 protein and its 80-kDa binding partner, CBP80, constitute a functional CBC (7–10). This nuclear complex binds cotranscriptionally to the monomethylated guanosine cap structure (m^7G) of RNA polymerase II-transcribed RNAs (7, 11, 12) and has been reported to play a role in diverse aspects of RNA metabolism: it increases the splicing efficiency of cap proximal introns (7, 13–15), positively affects the efficiency of 3′-end processing (16), and is required for the efficient transport of U snRNAs (9). We demonstrate that the incorporation of [α-^{32}P]GTP by CBP20 reflects the activation of the CBC and is correlated with its ability to bind capped RNA. A variety of growth factors and other cellular stimuli can activate the CBC under conditions that can give

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[1] The abbreviations used are: CBC, cap-binding complex; NGF, nerve growth factor; EGF, epidermal growth factor; TBS, Tris-buffered saline; DTT, dithiothreitol; AMP-PNP, adenosine 5′-(β,γ-iminotriphosphate); GMP-PNP, guanosine 5′-(β,γ-iminotriphosphate); PAGE, polyacrylamide gel electrophoresis; GST, glutathione *S*-transferase; HA, hemagglutinin; eIF, eukaryotic initiation factor; snRNA, small nucleotide RNA.

rise to a stimulation of the splicing of precursor mRNAs in an *in vitro* assay system. The implications of CBP20 functioning as a novel end point in signal transduction highlight the importance of RNA metabolism in regulated cell growth.

EXPERIMENTAL PROCEDURES

Cell Culture Conditions—Rat pheochromocytoma (PC12) cells were maintained in Dulbecco's modified Eagle's medium containing 5% fetal bovine serum, 10% horse serum, and antibiotic/antimycotic solution (Sigma). All other cell types, including HeLa, BHK21, and COS-7 cells, were maintained in Dulbecco's modified Eagle's medium with the addition of 10% fetal bovine serum and antibiotic/antimycotic solution. Prior to growth factor treatment, cells were switched to serum-free medium for 40 h. Growth factors (NGF (Life Technologies, Inc.), heregulin β1 (residues 177–244; a generous gift from Dr. Mark Sliwkowski, Genentech), and EGF (Calbiochem) or 25% fetal bovine serum) were then added to the serum-free medium in the concentrations and for the times indicated under "Results" at 37 °C. Following treatment, the growth factor-containing medium was removed, and the cells were washed twice with Tris-buffered saline (TBS; 25 mM Tris-Cl, pH 7.4, 140 mM NaCl, and 1.0 mM EDTA) and then lysed (see below). Cell cycle blocks were performed in HeLa cells. A G_0 block was achieved by switching to serum-free medium for 22–24 h. For G_1/S phase arrest, 2.5 mM thymidine was added to the growth medium for 22–24 h. 80 ng/ml nocodazole was added to the growth medium for 22–24 h to achieve arrest in M phase. After treatment, cells were collected, washed twice with TBS, and lysed. To challenge cells with UV irradiation, the medium was removed from serum-starved cells, and the cells were then exposed to UV light for 2 min. Following exposure, cells were replenished with serum-free medium and allowed to recover at 37 °C for the times indicated below.

Cell Fractionation and Nuclear Lysis—Tissue culture cells were washed twice on the plate with TBS and then lysed in a buffer containing Hanks' solution (20 mM Hepes, pH 7.4, 5 mM KCl, 137 mM NaCl, 4 mM $NaHCO_3$, 5.5 mM D-glucose, and 10 μM EDTA), 0.3% (v/v) Nonidet P-40, 1 mM sodium orthovanadate, 1 mM DTT, 1 mM phenylmethanesulfonyl fluoride, and 10 μg/ml each leupeptin and aprotinin. The lysate was then centrifuged at 800 rpm for 15 min at 4 °C. The supernatant was microcentrifuged for 10 min at 4 °C, and then the resulting supernatant was saved as the cytoplasmic fraction. The nuclear pellet was washed twice with an equal volume of Hanks' solution with 0.2% (v/v) Triton X-100 and centrifuged at 800 rpm for 15 min at 4 °C. The resulting pellet was treated as the purified nuclear fraction. The nuclei were then lysed in a buffer containing 50 mM Tris, pH 7.4, 1% (v/v) Triton X-100, 400 mM KCl, 1 mM sodium orthovanadate, 1 mM DTT, and protease inhibitors as described above. The samples were incubated on ice for 30 min and microcentrifuged for 10 min at 4 °C, and the supernatant was used as the whole nuclear fraction. For nuclear fractionation, nuclei were isolated from tissue culture cells, and nuclear membranes and nuclear soluble fractions were then prepared as described by Davis and Blobel (17) with some modification. The whole nuclear fraction was resuspended in 50 mM Tris-HCl, pH 7.4, 10% (w/v) sucrose, 1 mM sodium orthovanadate, 1 mM DTT, 1 mM $MgCl_2$, and protease inhibitors. DNase-I (5 mg/ml) and RNase A (1 mg/ml) were added, and the nuclei were then incubated for 15 min at 37 °C. Following the incubation with DNase-I, the nuclei were underlaid with 30% sucrose and then subjected to centrifugation in a swinging bucket rotor for 10 min at 20,000 × *g* to generate a soluble nuclear fraction and a nuclear membrane fraction.

Photoaffinity Labeling with [α-^{32}P]GTP—Photoaffinity labeling of cellular proteins with [α-^{32}P]GTP was performed as described previously (18). In brief, the UV cross-linking reaction was carried out in a buffer containing 50 mM Hepes, pH 7.4, 2 mM EGTA, 1 mM DTT, 20% (v/v) glycerol, 100 mM NaCl, and 500 μM AMP-PNP. Samples (20 μl) prepared from the cell fractionation procedures, described above, were incubated for 10 min at room temperature with an equal volume of cross-linking buffer containing [α-^{32}P]GTP (2–3 μCi/sample, 3000 Ci/mmol; NEN Life Science Products) in a 96-well, non-tissue culture-treated plate. The samples were then placed in an ice bath and irradiated with UV light (254 nm) for 15 min. After irradiation, samples were mixed with 5× Laemmli buffer and boiled. SDS-PAGE was performed using 15% acrylamide gels. The gels were then typically silver-stained and dried, and autoradiography was performed (typically overnight) using Kodak X-Omat XAR-5 film at −80 °C. To perform competition experiments, competing nucleotides (m^7GpppG and GpppG (New England Biolabs Inc.) and m^7GTP and GTP (Sigma)) were added to the sample prior to the addition of the [α-^{32}P]GTP-containing cross-linking buffer. This buffer did not contain AMP-PNP. The samples were then subjected to UV cross-linking as described above.

Purification of an 18-kDa Protein from Bovine Retinal Tissue That Incorporates [α-^{32}P]GTP—Bovine retinas were obtained frozen from J. A. & W. L. Lawson Co. (Lincoln, NE). The retinas (typically 200/batch) were thawed in a buffer containing 50 mM Tris, pH 8.0, 25 mM KCl, 5 mM $MgCl_2$, and protease inhibitors as described for cell lysate preparations and then homogenized with a motor-driven Dounce homogenizer. The homogenate was centrifuged at 2500 rpm in a swinging bucket rotor to yield a crude nuclear pellet. The nuclei were purified from this crude preparation using the method described by Blobel and Potter (19), and the soluble nuclear contents were then extracted as described above. The 18-kDa activity was precipitated using 40–75% ammonium sulfate, resuspended in 3–5 ml of Buffer A (50 mM Tris, pH 8.0, 100 mM NaCl, 10 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, 20 mM KCl), and loaded onto a fast protein liquid chromatography Superdex-200 Highload 16/60 column as described above. The purification of this activity was monitored by both silver staining and UV cross-linking to [α-^{32}P]GTP. The fractions eluted from the Superdex-200 column were assayed for [α-^{32}P]GTP incorporation into the 18-kDa protein, and six peak fractions (eluting with molecular masses of ~100–150 kDa) were pooled in a final volume of 12 ml and loaded directly onto a fast protein liquid chromatography ion-exchange Mono Q 5/5 column (Amersham Pharmacia Biotech) equilibrated in Buffer A minus KCl. Bound proteins were eluted from the Mono Q 5/5 column with a 28-ml linear gradient of 100–500 mM NaCl. [α-^{32}P]GTP-incorporating activity eluted from the Mono Q 5/5 column with ~300 mM NaCl in a volume of 5 ml. Peak activity as assayed by [α-^{32}P]GTP incorporation was eluted from the Mono Q column and applied directly to a Bio-Gel HPHT hydroxylapatite column (Bio-Rad) equilibrated in 10 mM potassium phosphate, pH 6.8, 2.5 mM $MgCl_2$, 0.01 mM $CaCl_2$, and 1 mM DTT. Bound proteins were then eluted, first by stepping the potassium phosphate to 100 mM and then by a 20-ml linear gradient of 100–300 mM potassium phosphate. Peak activity as assayed by the light-catalyzed incorporation of [α-^{32}P]GTP was found to elute with ~250 mM phosphate.

Cloning and Expression of Recombinant CBP20—CBP20 was cloned by polymerase chain reaction from HeLa cell cDNA (a generous gift from Dr. Wannian Yang, Cornell University). 5′- and 3′-primers were designed using the published sequence for *Homo sapiens* CBP20 (GenBank™ accession P52298), and the *CBP20* gene was then amplified from the HeLa cell cDNA using 40 polymerase chain reaction cycles (1 min at 94 °C, 1 min at 55 °C, and 1 min at 72 °C). The 470-base product was inserted into a cloning vector (pCR2.1) using a TA cloning kit (Invitrogen) and then subcloned into the mammalian expression vector pcDNA3 (Invitrogen) and into the *Escherichia coli* expression vector pGEX-2TK.

E. coli cells transformed with the pGEX-2TK-CBP20 vector were grown in a 1-liter culture, and expression of glutathione *S*-transferase (GST)-CBP20 protein was induced for 3 h using isopropyl-β-D-thiogalactopyranoside. Following induction, the cells were pelleted by centrifugation (5000 rpm for 10 min in a JA-10 rotor). The harvested cells were resuspended in 15 ml of 50 mM Tris-HCl, pH 8.0, 50 mM EDTA, 1 mM DTT, and protease inhibitors (as described above) and then lysed using 15 mg of lysozyme, followed by the addition of 200 mM $MgCl_2$ and 1 mg of DNase-I. Following centrifugation (100,00 × *g* for 30 min at 4 °C), the supernatant was incubated with glutathione-agarose beads for 1 h at 4 °C to bind the GST-CBP20 protein. Glutathione-agarose-bound CBP20 was washed with 50 mM Tris-HCl, pH 8.0, 0.5% (v/v) Triton X-100, 200 mM KCl, and 1 mM DTT and then stored in a buffer containing 50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1 mM DTT, 10 μM GTP, and protease inhibitors. GST-CBP20 was eluted from the glutathione-agarose beads using 10 mM glutathione, pH 8.0, and the GST moiety was cleaved from CBP20 by the addition of 500 units of thrombin for 30 min at room temperature.

Using the LipofectAMINE protocol (Life Technologies, Inc.), a hemagglutinin-tagged form of CBP20 (HA-CBP20) was transiently transfected into BHK21 cells according to the manufacturer's directions. Following a 5-h incubation with serum-free medium containing the lipid-DNA complex, the medium was removed and replaced with medium containing 10% fetal bovine serum. Cells were allowed to grow in the presence of serum for ~20 h and were then switched to serum-free medium for 40 h prior to stimulation with serum.

Immunoprecipitation and Western Immunoblotting—A polyclonal antibody generated against recombinant CBP80 (αCBP80) was prepared as described previously (7). Cytosolic and nuclear lysates were prepared as described above. Prior to immunoprecipitation, the cytosolic lysate was adjusted to 100 mM NaCl, and the nuclear lysate was

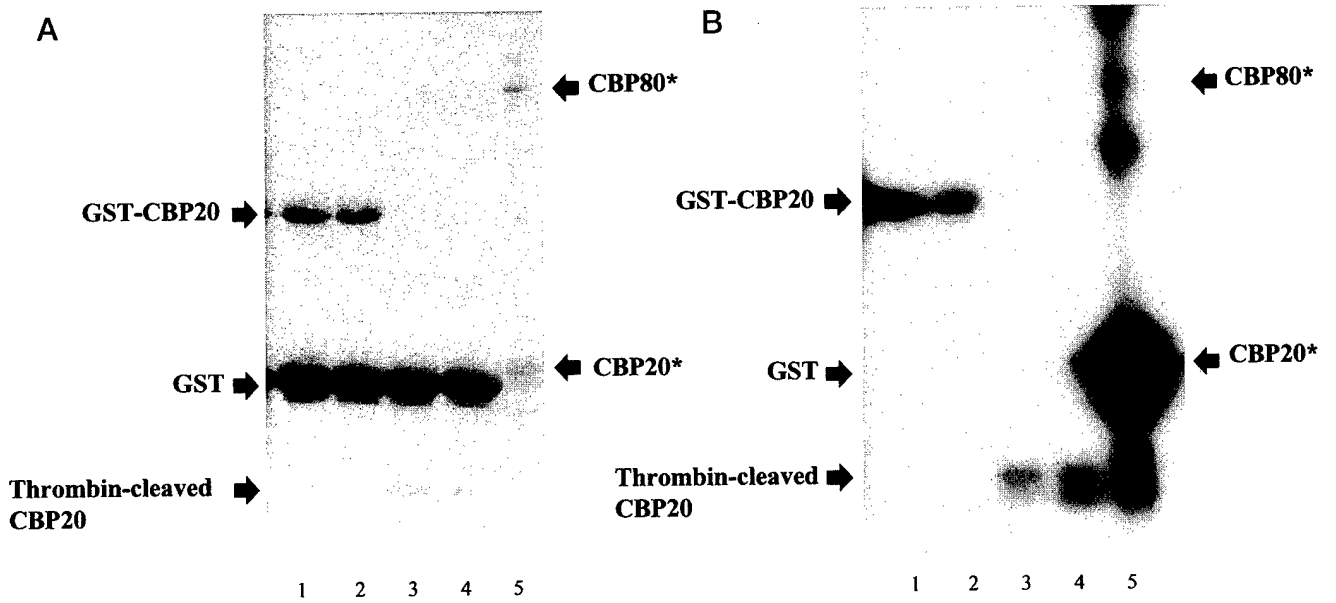

FIG. 1. **Recombinant *E. coli*-expressed CBP20 incorporates [α-^{32}P]GTP in a photoaffinity labeling assay.** CBP20 was expressed and purified from *E. coli* as a GST fusion protein. GST-CBP20 (*lanes 1* and *2*), free CBP20 (the GST was cleaved with thrombin) (*lanes 3* and *4*), and *E. coli*-expressed CBC (complex proteins designated as *CBP80** and *CBP20**) (*lane 5*) were then assayed for their ability to incorporate [α-^{32}P]GTP. Following UV cross-linking, proteins were separated by 15% SDS-PAGE and visualized by Coomassie Blue staining (*A*) and autoradiography (*B*).

diluted 3-fold with 50 mM Tris-HCl, pH 8.0, 1 mM DTT, and 1 mM sodium orthovanadate. The lysates were then allowed to incubate at 4 °C for 1 h, with or without the addition of 5 μl of 12CA5 monoclonal antibody or αCBP80 polyclonal antibody. Following the first incubation, 40 μl of protein A-Sepharose beads were added to each sample, and the samples were incubated for another hour at 4 °C. The samples were then centrifuged, and the immunoprecipitated pellets were washed four times with 50 mM Tris-HCl, pH 8.0, 133 mM KCl, 0.33% Triton X-100, 1 mM DTT, and 1 mM sodium orthovanadate. The resulting immunoprecipitated pellets were resuspended in 20 μl of UV cross-linking buffer and were incubated with [α-^{32}P]GTP and UV cross-linked as described above.

For Western blot analysis, proteins were transferred to polyvinylidene difluoride membranes following SDS-PAGE. The polyvinylidene difluoride membranes were blocked with 2.5% (w/v) bovine serum albumin in TBS plus 0.1% Tween 20 for 1 h at room temperature. After blocking, the membranes were incubated with either 12CA5 or αCBP80 antibody for 1 h at room temperature, washed with several changes of TBS and 0.1% Tween 20, and incubated for 30 min at room temperature with sheep anti-rabbit or sheep anti-mouse horseradish peroxidase-conjugated antibody (Amersham Pharmacia Biotech) as appropriate. Immunolabeling was detected by enhanced chemiluminescence (ECL, Amersham Pharmacia Biotech) according to the manufacturer's instructions.

RNA Binding Assays—UV cross-linking was done essentially as described by Rozen and Sonenberg (20), except that the RNA probe was transcribed from *Bam*HI-cleaved pBluescript II KS with T3 RNA polymerase (Promega).

Pre-mRNA Splicing Reactions—Splicing extracts were prepared from HeLa cells (serum-starved for 40 h prior to stimulation with 100 nM heregulin for 24 h) as described by Lee and Green (21). pBSAd1 precursor linearized by *Sau*3AI was transcribed using T3 RNA polymerase in the presence of m^{7}GpppG dinucleotide cap. Splicing reactions were then carried out as described by Izaurralde *et al.* (7). In brief, 60 μg of splicing extract were preincubated for 15 min at 30 °C with 1 mM $MgCl_2$. 5 mM creatine phosphate, 1.5 mM ATP, 2.5×10^4 cpm of labeled precursor mRNA, and an additional 1 mM $MgCl_2$ were then added in a final volume of 20 μl, and the reactions were incubated for 2 h at 30 °C. Splice products were visualized by separation on a 10% denaturing polyacrylamide gel, followed by autoradiography.

RESULTS

The overall goal of these studies was to identify nuclear activities that could represent novel downstream targets in receptor-coupled signaling pathways. One of the assays we used to identify such activities was the photocatalyzed incorporation of [α-^{32}P]GTP into nuclear proteins. The rationale for this approach was that it would provide a very sensitive assay for identifying guanine nucleotide-binding activities in the nucleus, in a manner analogous to the use of phosphorylation assays to identify growth factor-sensitive phosphosubstrates. Using this assay, we identified an 18-kDa protein that strongly incorporated [α-^{32}P]GTP in serum-treated but not serum-starved cells (see below). We found this activity to be exclusively nuclear and present in every cell line we examined, including HeLa, PC12, COS-7, and BHK21 cells, as well as in various mammary epithelial cells. A similar activity was also observed in the yeast *Saccharomyces cerevisiae*.

A purification scheme was developed using bovine retinal nuclei, which were a particularly rich source of this 18-kDa nuclear activity. A series of three chromatography steps resolved the activity, as assayed by [α-^{32}P]GTP incorporation, from the majority of contaminating low molecular mass proteins (see "Experimental Procedures"). These steps also resolved an 80-kDa protein (designated p80), detected by silver staining, which co-purified with the 18-kDa activity. This putative protein complex was reminiscent of the nuclear CBC, as the CBC comprises an 18-kDa nuclear protein, CBP20 (for cap-binding protein 20), stably complexed with an 80-kDa protein, designated CBP80. The formation of the CBP20-CBP80 heterodimer enables the CBC to bind a guanine derivative, the 7-methylguanosine cap structure (m^{7}GpppN), on RNAs transcribed by RNA polymerase II (7–10). The similarities between the 18-kDa nuclear activity and CBP20 (both in complex formation and substrate binding) led us to investigate whether the CBC was a nuclear target for extracellular signals.

First, we assayed directly the ability of recombinant *E. coli*-expressed CBP20 to incorporate [α-^{32}P]GTP. Fig. 1*A* shows GST-CBP20, thrombin-cleaved CBP20, and the complexed CBC proteins (His-tagged CBP20 (9) and CBP80 (7)) as visualized by staining with Coomassie Blue. Fig. 1*B* shows that the recombinant CBP20 proteins were all capable of incorporating [α-^{32}P]GTP in a photoaffinity labeling assay. This activity was greatly enhanced by the presence of CBP80 (see *lane 5*), consistent with previous studies that have demonstrated that complex formation between CBP20 and CBP80 is necessary for

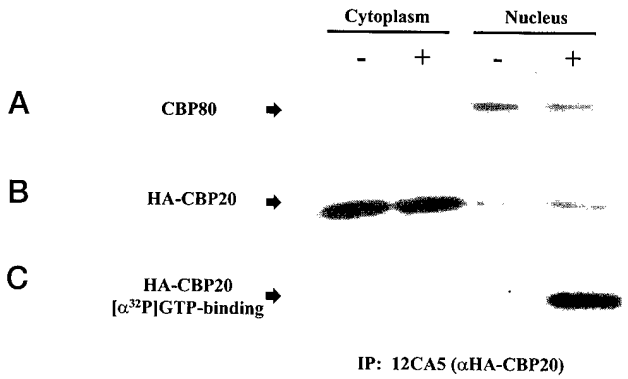

FIG. 2. **Serum-dependent incorporation of [α-^{32}P]GTP into recombinant HA-CBP20 expressed in BHK21 cells.** Human CBP20 was cloned by polymerase chain reaction from HeLa cell cDNA and then subcloned into the mammalian expression vector pcDNA3 to express a HA-tagged form of the protein. BHK21 cells were transiently transfected with HA-CBP20 (using 8 μg of DNA/100-mm plate of BHK21 cells). The transfected cells were serum-starved for 40 h (−) and then stimulated with 25% fetal bovine serum (+) for 1.5 h. HA-CBP20 was immunoprecipitated (*IP*) from cytosolic or nuclear lysates using 12CA5 monoclonal antibody. Immunoprecipitates were then assayed for [α-^{32}P]GTP incorporation. Proteins were separated by 15% SDS-PAGE and transferred to Immobilon for Western blot analysis and autoradiography. *A* shows the CBP80 protein co-immunoprecipitating with HA-CBP20 from the nuclear lysates as detected by Western blotting using CBP80 antiserum. *B* is a Western blot using 12CA5 antibody to detect immunoprecipitated HA-CBP20 from cytosolic and nuclear lysates. The [α-^{32}P]GTP incorporation corresponding to immunoprecipitated HA-CBP20 is shown in *C*.

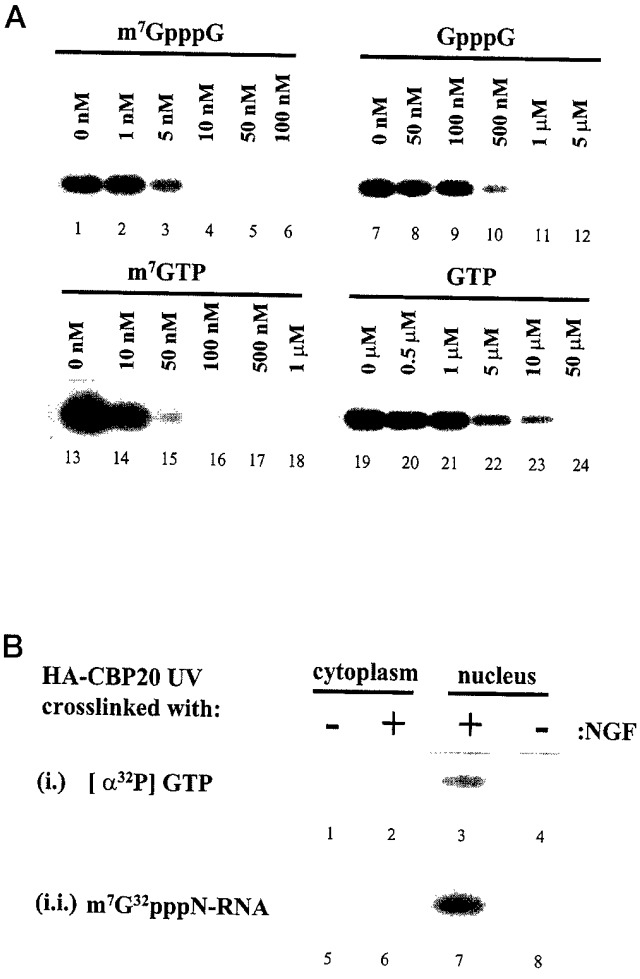

FIG. 3. **Incorporation of [α-^{32}P]GTP into CBP20 reflects an m^{7}GpppN-capped RNA binding event by the CBC that can be growth factor-regulated.** *A*, [α-^{32}P]GTP incorporation into PC12 cell CBP20 is blocked by the addition of RNA cap analogs. Nuclear extracts were prepared from PC12 cells growing asynchronously in culture. 200 μg of lysate were then immunoprecipitated with 5 μl of αCBP80 antiserum. The immunoprecipitates were assayed for [α-^{32}P]GTP incorporation into CBP20 in the presence of m^{7}GpppG (0, 1, 5, 10, 50, and 100 nM (*lanes 1–6*, respectively)), GpppG (0 nM, 50 nM, 100 nM, 500 nM, 1 μM, and 5 μM (*lanes 7–12*, respectively)), m^{7}GTP (0 nM, 10 nM, 50 nM, 100 nM, 500 nM, and 1 μM (*lanes 13–18*, respectively)), and GTP (0, 0.5, 1, 5, 10, and 50 μM (*lanes 19–24*, respectively)). Following cross-linking, proteins were separated by 15% SDS-PAGE; the gel was dried; and autoradiography was performed. *B*, CBP20 binds capped RNA in a growth factor-dependent manner. PC12 cells stably expressing HA-CBP20 were serum-starved (−) and then treated with 100 ng/ml NGF for 1 h (+). After preparation of cytosolic and nuclear lysates, HA-CBP20 was immunoprecipitated from the lysates and assayed in the presence of either [α-^{32}P]GTP (*lanes 1–4*) or m^{7}G^{32}pppN-capped RNA (*lanes 5–8*).

capped RNA binding. The GST control did not show any cross-linking to [α-^{32}P]GTP.

We next examined whether the ability of CBP20 to incorporate [α-^{32}P]GTP could be regulated in response to serum. BHK21 cells were transiently transfected with a HA-tagged CBP20 construct. Following 40 h of serum starvation, the cells were stimulated with 25% fetal bovine serum for 1.5 h, and HA-CBP20 was immunoprecipitated from cytosolic and nuclear lysates prepared from either serum-starved or stimulated cells. The immunoprecipitates were then assayed for the photocatalyzed incorporation of [α-^{32}P]GTP into CBP20. HA-CBP20 was present in both the cytosolic and nuclear fractions (Fig. 2*B*), and CBP80 co-immunoprecipitated with nuclear localized HA-CBP20 equally well under conditions of either serum starvation or stimulation (Fig. 2*A*). The large percentage of HA-CBP20 localized to the cytosol is presumably the result of its overexpression. Nuclear CBP20 demonstrated a marked serum-dependent incorporation of [α-^{32}P]GTP (Fig. 2*C*).

Given that the m^{7}GpppN RNA cap structure is a known substrate for the CBC, the stimulated incorporation of [α-^{32}P]GTP into CBP20 may reflect an enhanced ability of the CBC to bind the cap structure on RNA. To address this issue, we first examined the relative binding affinities of the CBC for different cap analogs by testing their ability to inhibit the incorporation of [α-^{32}P]GTP into CBP20. PC12 cell nuclear lysates were immunoprecipitated with antibodies generated against CBP80 (*i.e.* the binding partner of CBP20) (7), and the immunoprecipitates were then assayed for photocatalyzed incorporation of [α-^{32}P]GTP into CBP20 in the absence and presence of RNA cap analogs or GTP. CBP20 proteins that co-immunoprecipitated with CBP80 could be efficiently labeled with [α-^{32}P]GTP. This activity was strongly inhibited by the addition of low concentrations of cap analogs to the [α-^{32}P]GTP cross-linking assay and yielded the following binding specificity: m^{7}GpppG > m^{7}GTP > GpppG > GTP (Fig. 3*A*). Indeed, the m^{7}GpppG analog competed with [α-^{32}P]GTP for binding to CBP20 ~1000 times more effectively than GTP, suggesting that the CBC most likely binds RNA, rather than GTP, in cells.

We further examined whether the CBC shows a regulated binding to capped RNAs using a PC12 cell line that stably expresses HA-tagged CBP20. Following starvation, these cells were stimulated with NGF. HA-CBP20 was then immunoprecipitated from the cytoplasmic and nuclear lysates and assayed for the incorporation of either [α-^{32}P]GTP (Fig. 3*B*, *upper panel*) or m^{7}G^{32}pppN-capped RNA (*lower panel*). Both substrates were incorporated into nuclear HA-CBP20 strictly in a growth factor-dependent manner. Thus, these findings indicate that the growth factor-stimulated incorporation of [α-^{32}P]GTP into CBP20 accurately reflects the activation of the CBC, such that it is induced to bind m^{7}GpppN-capped RNAs.

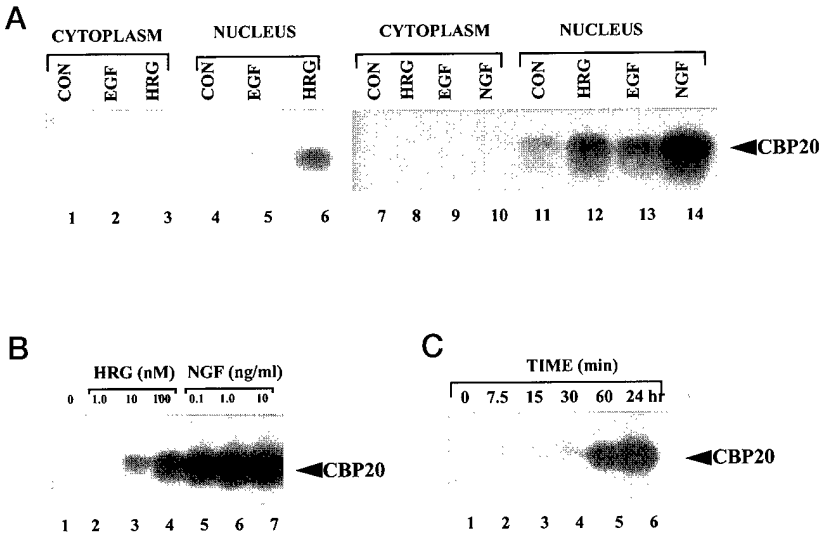

FIG. 4. **Characterization of the growth factor-induced capped RNA-binding activity of the CBC (assayed by CBP20 [α-^{32}P]GTP incorporation).** *A*, HeLa cells (*lanes 1–6*) were serum-starved (control (*CON*); *lanes 1* and *4*) and then treated with 100 ng/ml EGF (*lanes 2* and *5*) or 30 nM heregulin (*HRG*; *lanes 3* and *6*) for 15 min at 37 °C. PC12 cells (*lanes 7–14*) were serum-starved (control; *lanes 7* and *11*) and then treated with 30 nM HRG (*lanes 8* and *12*), 100 ng/ml EGF (*lanes 9* and *13*), or 100 ng/ml NGF (*lanes 10* and *14*). The cells were lysed, separated into cytoplasmic (*lanes 1–3* and *7–10*) and whole nuclear (*lanes 4–6* and *11–14*) fractions, and assayed for [α-^{32}P]GTP incorporation into CBP20 using 45 μg of protein from cell lysates. *B*, a dose-response experiment was performed with the addition of either heregulin (*lanes 2–4*) or NGF (*lanes 5–7*) to serum-starved PC12 cells (control; *lane 1*) for 30 min at 37 °C. For each dose of heregulin or NGF, 50 μg of total nuclear lysate protein were assayed for the incorporation of [α-^{32}P]GTP into CBP20, and then 15% SDS-PAGE was performed. The resulting gel was dried and exposed to x-ray film for 5–15 h. *C*, a time course of 100 ng/ml NGF treatment was performed in serum-starved PC12 cells (control; *lane 1*) with NGF addition for 7.5 min (*lane 2*), 15 min (*lane 3*), 30 min (*lane 4*), 60 min (*lane 5*), or 24 h (*lane 6*). 50 μg of protein from the nuclear lysates were assayed for incorporation of [α-^{32}P]GTP into CBP20, followed by 15% SDS-PAGE and autoradiography overnight.

We took further advantage of the high sensitivity of the [α-^{32}P]GTP incorporation assay to examine the abilities of different growth factors to activate the endogenous CBC. Fig. 4*A* (*left panel*) shows the results obtained when HeLa cells were first serum-starved and then treated with EGF and heregulin (the ligand for the Neu-ErbB2/ErbB3 and Neu-ErbB2/ErbB4 heterodimers (22, 23)). Endogenous CBP20 present in nuclear lysates from HeLa cells was strongly stimulated to incorporate [α-^{32}P]GTP by heregulin as well as, to a lesser extent, by EGF. Similarly, in PC12 cells, endogenous CBP20 present in nuclear lysates was activated by growth factors (Fig. 4*A*, *right panel*). In this case, the incorporation of [α-^{32}P]GTP into CBP20 was most strongly stimulated by NGF (as observed in Fig. 3*B*), followed by heregulin and then EGF. Fig. 4*B* shows that in all cases, the growth factor-stimulated activation of CBP20 was dose-dependent.

In our initial experiments, the incorporation of [α-^{32}P]GTP into CBP20 was assayed after relatively short periods of growth factor treatment (~15 min). Although this was sufficient to detect incorporation of the radiolabeled GTP, more complete time course experiments indicated that near maximal incorporation occurred following treatment with growth factors for 1 h. An example for PC12 cells is shown in Fig. 4*C*. In this experiment, serum-starved PC12 cells were challenged with 100 ng/ml NGF for increasing time periods, up to 24 h. The results show that near maximal incorporation of [α-^{32}P]GTP into CBP20 was observed after ~1 h of growth factor addition and that this level of incorporation was maintained through 24 h. A similar time course was obtained when PC12 cells were treated with heregulin (data not shown).

Nuclear lysates from asynchronously growing cells also contain activated CBP20, suggesting that the growth factor regulation of the CBC activity may be associated with a particular phase of the cell cycle. This is illustrated in Fig. 5*A*. HeLa cells were arrested in G_0 phase by serum starvation, in G_1/S phase by thymidine addition, and in M phase by nocodazole treatment. Cytoplasmic and nuclear fractions were then prepared (or a mitotic pellet was prepared in the case of M phase-arrested cells), and the resulting lysates were assayed for the ability of CBP20 to incorporate radiolabeled GTP. We found that CBP20 did not incorporate [α-^{32}P]GTP in cells arrested in either G_0 or M phase of the cell cycle. However, CBP20 strongly incorporated [α-^{32}P]GTP in HeLa cells arrested in G_1/S phase. Thus, the activation of the CBC appears to be sensitive to cell cycle-dependent as well as growth factor-dependent regulation.

To determine whether the CBC might respond to a broader range of stimuli, we assayed the ability of CBP20 to incorporate radiolabeled GTP under conditions of cellular stress. PC12 cells were first serum-starved and then exposed to UV radiation for 2 min. Following this exposure, the cells were allowed to recover for 30 min or 1 h, and then endogenous CBP20 was assayed for its ability to incorporate [α-^{32}P]GTP. Fig. 5*B* shows that CBP20 was strongly stimulated to incorporate radiolabeled GTP in cells that had been UV-irradiated. We found a similar stress activation of endogenous CBP20 in COS-7 and HEK-293 cells (data not shown).

Stress response pathways have been shown to be mediated by the low molecular mass GTP-binding proteins Cdc42 and Rac and to culminate in transcriptional activation through the stimulation of the nuclear mitogen-activated protein kinases JNK1 and p38/HOG1 (3–5, 24). Thus, we examined whether the transient expression of activated Cdc42 would result in a growth factor-independent activation of the CBC. The results in Fig. 5*C* indicate that this is the case. We found that the transient expression of either a GTPase-defective Cdc42 mutant (Cdc42 Q61L) or a transforming Cdc42 mutant that is capable of undergoing the spontaneous exchange of GTP for GDP (Cdc42F28L) strongly activated CBP20, whereas expression of wild-type Cdc42 showed no activation. We also have found that expression of V12-Ras stimulates the incorporation of [α-^{32}P]GTP into CBP20 as well as activated Rac and RhoA (data not shown), although thus far, Cdc42 appears to be the most effective activator.

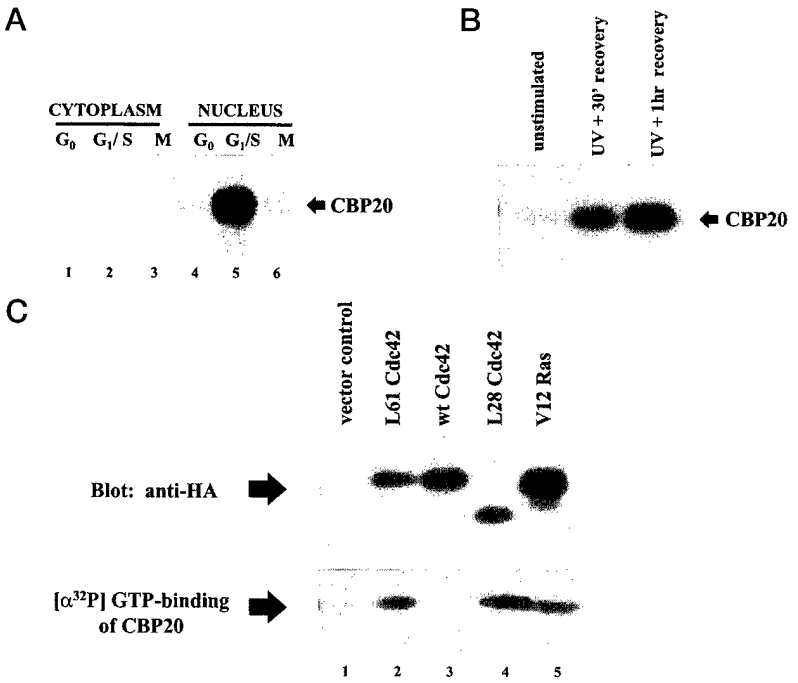

FIG. 5. **The CBC shows a cell cycle- and cell stress-dependent activation and can be activated by the low molecular mass GTP-binding proteins Cdc42 and Ras.** *A*, HeLa cells were arrested in G_0 phase by serum starvation (*lanes 1* and *4*), in G_1/S phase by 2.5 mM thymidine addition (*lanes 2* and *5*), and in M phase with 80 ng/ml nocodazole (*lanes 3* and *6*). The cells were then separated into cytoplasmic (*lanes 1–3*) and whole nuclear (or a mitotic pellet was prepared for M phase arrest) (*lanes 4–6*) fractions, and then for each fraction, 50 μg of protein were assayed for [α-^{32}P]GTP incorporation into CBP20, followed by 15% SDS-PAGE and autoradiography. *B*, PC12 cells were serum-starved and then exposed to UV light for 2 min. Following exposure, cells were replenished with serum-free medium and allowed to recover for 30 min or 1 h. Cells were then harvested; nuclear lysates were prepared; and 50 μg of nuclear lysate protein were assayed for [α-^{32}P]GTP incorporation into CBP20 by 15% SDS-PAGE and autoradiography. *C*, HeLa cells were transiently transfected with GTPase-defective Cdc42 Q61L (*L61 Cdc42*), wild-type (*wt*) Cdc42, constitutively active Cdc42 F28L (*L28 Cdc42*), or GTPase-defective Ras G12V (*V12 Ras*) for 24 h, followed by serum starvation for 40 h. The cytoplasmic lysates were analyzed for expression of the transfected proteins by Western blotting using an anti-HA antibody directed against the HA tag on the recombinant GTP-binding proteins (*upper panel*). The nuclear lysates were assayed for [α-^{32}P]GTP incorporation into CBP20 (*lower panel*).

Taken together, these data suggest that the ability of the CBC to bind RNA cap structures is a tightly regulated process. Previous work by others has defined a role for CBC binding to capped RNAs in important RNA metabolic processes, including pre-mRNA splicing (7, 13–15), U snRNA export (9), and 3′-end processing (16). The ability of growth factors to stimulate the capped RNA-binding activity of the CBC suggests that those metabolic processes that benefit from the recognition of the RNA cap by the CBC (such as pre-mRNA splicing) will also be subject to extracellular regulation. To test this prediction, splicing extracts were prepared from HeLa cells that were either serum-starved or starved and then stimulated with heregulin for 24 h (*i.e.* conditions that lead to maximal stimulation of CBP20 activity in nuclear lysates (see Fig. 4*B*)). Creatine phosphate, ATP, and m^7GpppG-capped precursor adenovirus mRNA were added to initiate splicing (see "Experimental Procedures"). Extracts prepared from quiescent cells were not competent to splice the m^7GpppG-capped precursor RNA (Fig. 6). However, splicing of the m^7GpppG-capped RNA was markedly stimulated in extracts prepared from heregulin-treated cells and was ~5-fold higher than the splicing of a nonspecific ApppG-capped RNA probe by the same extract (data not shown). These results indicate that under conditions where growth factor signaling activates CBC, there is a corresponding stimulation in capped precursor mRNA splicing. Because we also observed some increase in ApppG-capped RNA splicing, the possibility exists that other targets, perhaps acting in conjunction with the CBC, may be important in mediating the observed growth factor effect in cap-dependent RNA splicing. Thus, cap-dependent RNA splicing, in addition to CBC-capped RNA binding, is a functional end point for growth factor-coupled signaling pathways leading to the nucleus.

DISCUSSION

The original goal of these studies was to identify novel nuclear activities that were susceptible to growth factor regulation to further our understanding of how growth factors exert their effects in the nucleus. Using a photoaffinity labeling approach to detect nuclear proteins that specifically incorporate [α-^{32}P]GTP, we detected an 18-kDa nuclear activity that was highly sensitive to the addition of growth factors to G_0 phase-arrested cells. The fundamental role of this activity in cell growth regulation is underscored by its response to growth factors, its specific association with the G_1/S phase of the cell cycle, its activation under conditions of cell stress, and the fact that we have found this activity in every cell and tissue type examined thus far. It was therefore interesting to find that the 18-kDa activity corresponds to the RNA cap-binding protein CBP20, suggesting a necessity for a regulated nuclear cap binding event in cell growth control.

The m^7G(5′)ppp(5′)N cap structure on RNAs transcribed by RNA polymerase II has been known for some time to be important for the stability of these RNAs (25, 26) and to facilitate different aspects of RNA metabolism, including translation initiation, pre-mRNA splicing, and nuclear transport. In recent years, CBP20 and its 80-kDa binding partner, CBP80 (collectively termed CBC), have been identified as the protein complex that binds to the cap structure in the nucleus and mediates the cap-dependent enhancement of pre-mRNA splicing and export of U snRNAs (7, 9). To our knowledge, this is the

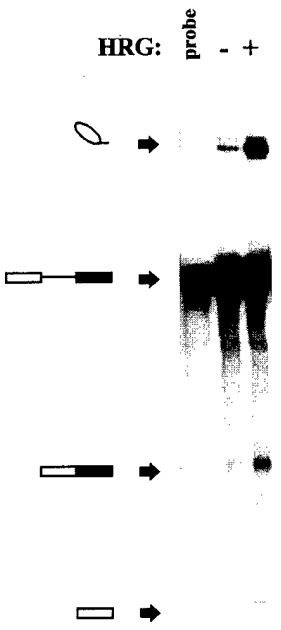

FIG. 6. **Splicing of m^{7}GpppN-capped RNAs is initiated by growth factor receptor-coupled signal transduction to the nucleus.** Splicing extracts were prepared from HeLa cells that were either serum-starved or starved and then stimulated with heregulin (*HRG*) (100 nN) for 24 h. These lysates were then assayed for their ability to support splicing of an m^{7}GpppG-capped pre-mRNA probe. The mature splice products and intermediates of the splicing reaction are indicated diagrammatically on the left. The results shown are representative of three experiments.

first report describing a regulated binding activity by the CBC and thus implies that RNA metabolic processes ascribed to the CBC will be regulated as well. This is supported by our finding that growth factors regulate the *in vitro* splicing of precursor mRNA in nuclear lysates from HeLa cells.

An understanding of the signaling processes that lead to CBC activation could shed light on how mitogens influence gene expression by modulating RNA metabolism. All indications are that the CBC may receive inputs from multiple pathways. The Ras-Raf-MEK-extracellular receptor-activated kinase signaling cascade is one pathway that is central to mediating growth factor effects in the nucleus, and we have observed that expression of oncogenic Ras G12V in cells results in an activation of the CBC. Stress-activated signaling pathways also induce CBC activation. There are a number of lines of evidence that indicate that signaling pathways stimulated by Rho-like GTP-binding proteins (*e.g.* Cdc42 and Rac) both participate in cellular stress responses (4–7, 27) and are under growth factor control (25–27). In fact, we have found that activated forms of Cdc42 give rise to an effective activation of the CBC. Given that Cdc42 has been suggested to input into rapamycin-sensitive pathways involving FRAP (FKB12/rapamycin-associated protein) by activating the p70 S6 kinase (27), it is interesting to consider whether the regulation of the CBC is linked to translational control. The cytosolic mRNA cap-binding protein eIF-4E, which plays a critical role in a number of mRNA translational events (29), is also susceptible to growth factor regulation. The phosphorylation of eIF-4E occurs in response to multiple growth factors (including NGF in PC12 cells) and cell cycle arrest (28, 30) and appears to occur downstream of multiple signaling pathways, including the extracellular receptor-activated kinase, c-Jun N-terminal kinase/stress-activated protein kinase, and p38 kinase pathways (29). In addition to its direct phosphorylation, the activity of eIF-4E is also regulated by two other growth factor-responsive factors, the eIF-4E-binding proteins 4E-BP1 and 4E-BP2 (30), and recently, 4E-BP1 has shown to be phosphorylated by the phosphatidylinositol 3-kinase-related kinase FRAP (31). Thus, it will be interesting to see if the cytosolic cap-binding protein eIF-4E and the CBC are similarly or even coordinately regulated through growth factor-initiated signals.

A growth factor-dependent phosphorylation of CBP20 could have a direct effect on its RNA cap-binding activity (similar to eIF-4E), although thus far, we have not been able to detect a growth factor-stimulated phosphorylation of CBP20 *in vivo*. The cellular levels of CBP20, its ability to bind CBP80, and its nuclear localization are not affected by growth factor stimulation (see Fig. 2). We are currently examining whether growth factors influence the interactions between the CBC and specific regulatory proteins to stimulate the binding of the CBC to capped RNA in a manner analogous to the growth factor-regulated interaction between eIF-4E and the 4E-BP proteins.

Our demonstration that the CBC is susceptible to extracellular regulation, in conjunction with the previously defined role for the CBC in RNA processing, makes the CBC an attractive candidate for translating growth factor signals into altered gene expression by affecting the metabolism of specific subsets of RNAs. However, given that the CBC affects both the processing and transport of RNAs transcribed by RNA polymerase II, the growth factor-dependent binding of the CBC to capped RNA may result in a general regulation of gene expression. The reduced ability of the CBC to bind capped RNAs in the absence of a growth factor signal could serve as a checkpoint for cell growth by guarding against the further processing of inappropriate or "leaky" transcripts. This suggests that altered levels and/or mutations of the CBC might be capable of deregulating cell growth. Future studies will be directed toward determining how growth factors influence different aspects of RNA processing (including precursor mRNA splicing and RNA export) through the CBC and how overexpression and/or mutation of the CBC impacts upon normal cell growth.

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Cdc42 Stimulates RNA Splicing via the S6 Kinase and a Novel S6 Kinase Target, the Nuclear Cap-binding Complex*

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Cdc42 is a low molecular weight GTP-binding protein that plays a key regulatory role in a variety of cellular activities. The importance of the coordination of different cell functions by Cdc42 is underscored by the fact that a constitutively active Cdc42 mutant induces cellular transformation. In this study, we describe a novel function for Cdc42: its ability to stimulate pre-messenger RNA splicing. This activity is dependent on cysteine 37 in the effector loop of Cdc42 but is not dependent on cell growth. A likely candidate protein for mediating the Cdc42 effects on pre-mRNA splicing is the nuclear RNA cap-binding complex (CBC), which plays a key role in an early step of cap-dependent RNA splicing. Activation of the CBC by Cdc42 can be inhibited by rapamycin. Additionally, phosphatidylinositol 3-kinase and the Cdc42 effector, pp70 S6 kinase, stimulate the RNA cap-binding activity of the CBC. S6 kinase may directly target the CBC *in vivo* as it can phosphorylate the 80-kDa subunit of the CBC, CBP80, at residues that are subject to a growth factor-dependent and rapamycin-sensitive phosphorylation *in vivo*. Together these data suggest the involvement of a Cdc42-S6 kinase pathway in the regulation of RNA splicing, mediated by an increase in capped RNA binding by the CBC, as well as raise the possibility that the effects of Cdc42 on cell growth may be due in part to its regulation of RNA processing.

AQ: A

Cdc42 is a low molecular weight GTP-binding protein of the Ras superfamily that coordinates multiple cellular events by a regulated binding and hydrolysis of GTP. The GTP-bound state is considered the active form of Cdc42, which interacts with downstream target/effector proteins and is responsible for signal propagation. To date, several effectors for Cdc42 have been described and appear to be necessary for mediating the regulation of a diversity of cellular functions. Transcriptional regulation (1–5), vesicular trafficking (6), and actin cytoskeletal arrangement and cell cycle progression (7, 8) all represent activities in which Cdc42 has been implicated. Additionally, it has been shown that Cdc42 plays an important role in cell growth control, as a constitutively active form of Cdc42, Cdc42 F28L, induces malignant transformation (9). However, the mechanisms responsible for Cdc42-mediated transformation are still not entirely clear. We have recently shown that an interaction between Cdc42 and the coatomer subunits of COP-coated vesicles is somehow involved in the transformation signal (6). However, there appears to be a requirement for other Cdc42 targets as well, as deletion of the insert region of Cdc42 blocks transformation (10) independent of any interaction with the coatomer complex. What this suggests is that Cdc42 may be orchestrating what appear to be distinct cellular functions in order to coordinate regulated cell growth. Therefore, an understanding of the different biological activities regulated by Cdc42 and the distinct effector pathways that mediate these activities will provide insight into how Cdc42 stimulates cell cycle progression and growth.

In a previous study, we observed that activated forms of Cdc42 were capable of stimulating the nuclear cap-binding complex (CBC)[1] to bind capped RNAs (11). In binding to RNAs containing a m^7G cap structure, the CBC has been implicated in a number of fundamental aspects of RNA processing including pre-mRNA splicing (12–15), U snRNA export (16), and polyadenylation (17). Importantly, the CBC has also been shown to be a nuclear end point for extracellular stimuli such as growth factors and cell stress stimulants (11). In this study we have investigated the possibility that Cdc42 might be involved in the signal regulation of RNA processing via the CBC. Specifically, we find that activated alleles of Cdc42 can stimulate splicing activity in a manner that is dependent on amino acid 37 of the effector loop. The inhibition of splicing activity, which occurs when the effector loop is mutated at position 37, is coincident with a loss of Cdc42-mediated transformation suggesting that RNA splicing may be coupled to cell growth regulation by Cdc42. As the CBC plays a pivotal role in cap-dependent pre-mRNA splicing, we have investigated the Cdc42 pathway leading to CBC activation and find that this pathway proceeds via the Cdc42 effector, pp70 S6 kinase (S6K). Rapamycin inhibits the Cdc42-induced activation of the CBC, whereas S6K, together with PI3 kinase, will induce the CBC to bind capped RNAs. Additionally, S6K can phosphorylate the CBC *in vitro* at a site that undergoes a rapamycin-sensitive, growth factor-dependent phosphorylation *in vivo*. Together, these data suggest a mechanism by which Cdc42 might influence pre-mRNA splicing, through the S6K-mediated activation of the CBC.

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MATERIALS AND METHODS

Cell Culture Conditions and Cell Lysis—HeLa and COS-7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum and antibiotic/antimycotic solution (Life Technologies, Inc.). Rat pheochromocytoma (PC12) cells were grown in the same medium with the addition of 5% horse serum, and NIH 3T3 cells

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[1] The abbreviations used are: CBC, cap-binding complex; PI3 kinase, phosphatidylinositol 3-kinase; DMEM, Dulbecco's modified Eagle's medium; HA, hemagglutinin; NGF, nerve growth factor; DTT, dithiothreitol; PCR, polymerase chain reaction; NLS, nuclear localization signal; GST, glutathione *S*-transferase; PAGE, polyacrylamide gel electrophoresis; FRAP, FK506-binding protein-12/rapamycin-associated protein kinase; eIF, eukaryotic initiation factor; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid.

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were grown in DMEM with 10% calf serum. Stable NIH 3T3 cell lines expressing either HA-tagged Cdc42 F28L,C37A or HA-tagged Cdc42 F28L,Y40C from the pJ4H vector were generated as described previously (9). Transfection experiments were performed according to the manufacturer's directions using the LipofectAMINE Plus reagent (Life Technologies, Inc.). Constructs for transient expression in mammalian cells include: pcDNA$_3$ HA-L61 Cdc42, pcDNA$_3$ HA-wild type Cdc42, pcDNA$_3$ HA-L28 Cdc42, pCMV myristoylated 110-kDa PI3 kinase catalytic subunit, pJ3H Myc-tagged S6K, and various forms of pcDNA$_3$ CTKT$_3$ CBP80 (see below). As indicated, HeLa cells and PC12 cells were switched to serum-free medium for 40–48 h or starved and treated with growth factors (NGF, 100 ng/ml (Life Technologies, Inc.); heregulin β1, 100 nM (residues 177–244; a generous gift from Dr. Mark Sliwkowski, Genentech)) prior to harvesting. Concentrated stocks of rapamycin and wortmannin (Sigma) were prepared in ethanol. Prior to addition, the appropriate volume of inhibitor was dispensed, the ethanol was evaporated, and the drug was resuspended in a small volume of DMEM. Cells were then treated with the inhibitor for 30 min at the concentrations indicated in the text. Cell fractionation and lysate preparation were as described previously (11).

To metabolically label PC12 cells, the cells were washed once with phosphate-free DMEM and then were incubated with phosphate-free DMEM containing 1.5 mCi/ml [^{32}P]orthophosphoric acid (150 mCi/ml, PerkinElmer Life Sciences) for 3 h followed by NGF and/or rapamycin as indicated in the text. Lysis buffers for this procedure included the addition of 50 mM β-glycerol phosphate and 50 mM NaF to the previously described cytosolic and nuclear lysis buffers. HeLa cells were transfected with pcDNA$_3$ CTKT$_3$ CBP80 constructs for 24 h, metabolically labeled with [^{32}P]orthophosphoric acid, and treated with heregulin. For these transfection experiments, whole cell lysates were prepared using a buffer that contained 0.5% Triton X-100 in Hanks' buffer (20 mM Hepes, pH 7.4, 5 mM KCl, 137 mM NaCl, 4 mM $NaHCO_3$, 5.5 mM D-glucose, 10 μM EDTA) with 1 mM DTT, 1 mM sodium orthovanadate, 50 mM β-glycerol phosphate, 50 mM NaF, and protease inhibitors.

Cloning of CBP80 and Expression of Recombinant Proteins—CBP80 was cloned from a human testis cDNA library (the library was a generous gift of Dr. Nena Winand, Cornell University) using 5′ and 3′ primers designed from the published sequence for hsCBP80 (GenBank™ accession number 1705654) and 40 PCR cycles (30 s at 94 °C, 30 s at 60 °C, and 3 min at 68 °C). The PCR fragment was subcloned into the baculovirus transfer vector pVL1393, and a recombinant CBP80 baculovirus was generated according to the manufacturer's directions (Pharminogen). CBP80 NLS mutants (CBP80 S2A, CBP80 S7A, CBP80 T21A,S22A, and CBP80 S7A,T21A,S22A) were made using PCR-based site-directed mutagenesis. The DNAs encoding wild type and mutant CBP80 proteins were then subcloned into pcDNA3, and the sequence encoding a KT_3 tag (38) was inserted at the 3′ end of the gene.

Recombinant CBP80 and a NLS mutant CBP80 (designated NLS2 CBP80, the virus was a generous gift of Dr. Iain Mattaj, EMBL, Heidelberg, Germany (23)) were generated by infecting 75-cm^2 tissue culture flasks, each containing 6×10^6 Sf21 cells, with 50 μl of the appropriate virus for 3 days. Infected Sf21 cells were then lysed using a buffer containing 50 mM Tris-HCl, pH 8.0, 0.4% CHAPS, 300 mM NaCl, 1 mM DTT, and protease inhibitors. The lysates were then cleared by centrifugation for 30 min at 40,000 rpm. CBP80 was purified from the lysate using GST-CBP20 (11) as an affinity column. The column containing CBP80 bound to GST-CBP20 was washed extensively with 50 mM Tris-HCl, pH 8.0, 0.5% Triton X-100, 200 mM KCl, and 1 mM DTT. The bound proteins were eluted with 10 mM glutathione in buffer D (20 mM Hepes, pH 8.0, 20% glycerol, 100 mM KCl, 0.2 mM EDTA, and 1 mM DTT). Thrombin was used to cleave the GST from CBP20, and the GST and free CBP20 were separated from the CBC on a Mono Q column (Amersham Pharmacia Biotech). The purified CBC fractions were then dialyzed against buffer D and stored at −80 °C.

Photoaffinity Labeling and in Vitro Kinase Assays—CBC cap-binding activity was assayed by measuring the incorporation of [α-^{32}P]GTP into CBP20 by UV cross-linking as established previously (11). For *in vitro* kinase assays, S6K (either immunoprecipitated from lysates prepared from COS-7 cells that had been transfected with pJ3H Myc S6K or 20 units of partially purified enzyme (Upstate Biotechnology, Inc.)) was incubated with CBC as indicated in the text, in a buffer containing 10 mM Hepes, pH 8.0, 20 μM ATP, 5 mM $MgCl_2$, and 10 μCi/sample [γ-^{32}P]ATP (3000 Ci/mmol, PerkinElmer Life Sciences), for various times at room temperature. The reactions were stopped by the addition of SDS-PAGE sample buffer, and the proteins were resolved by SDS-PAGE. Phosphorylated proteins were visualized by autoradiography.

Pre-mRNA Splicing Reactions—Splicing extracts were prepared as described by Lee and Green (39) from serum-starved (1% calf serum for 24 h) NIH 3T3 cells stably expressing different Cdc42 constructs. pBSAd1 precursor linearized by *Sau*IIA was transcribed using T3 RNA polymerase in the presence of m^{7}GpppG or ApppG dinucleotide cap (New England Biolabs). Splicing reactions were then carried out as described previously (11).

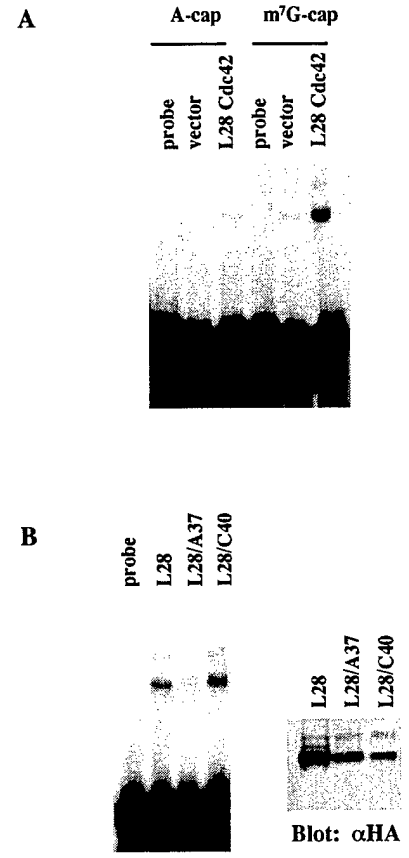

FIG. 1. **Activated Cdc42 induces cap-dependent RNA splicing in NIH 3T3 cells in a manner dependent on position 37 in the effector loop.** *A*, NIH 3T3 cells stably transfected with Cdc42 F28L or vector alone were reduced to 1% calf serum for 24 h, and then splicing extracts were prepared and analyzed for their ability to support the splicing of either a specific m^{7}GpppG-capped or nonspecific ApppG-capped probe. The lower diffuse band represents the unspliced precursor RNA probe, and the upper band is the lariat formation splice product. B, splicing experiments were performed using NIH 3T3 cells stably expressing Cdc42 F28L (designated *L28*), Cdc42 F28L,C37A (*L28/A37*), and Cdc42 F28L,Y40C (*L28/C40*). The *left-hand panel* shows the results from the splicing experiment, and the *right hand panel* depicts a Western blot of the Cdc42 expression levels using an antibody against the HA-tag of the expressed Cdc42 protein.

RESULTS AND DISCUSSION

Previously, we have demonstrated that activated forms of Cdc42 can promote the nuclear CBC to bind capped RNA substrates (11). One of the important functional outcomes of this binding event is an enhancement of capped RNA splicing (12). This leads to the intriguing suggestion that Cdc42-stimulated signaling pathways can promote cap-dependent RNA splicing, a previously unsuspected function for Cdc42. To test this possibility, splicing lysates were prepared from NIH 3T3 cells that were stably transfected with a constitutively active form of Cdc42, Cdc42 F28L (9), or vector alone, after a 24-h growth period in low serum. The activity of these splicing lysates was then assessed in an *in vitro* splicing assay using either a specific m^{7}GpppG-capped probe or a nonspecific ApppG-capped probe. As shown in Fig. 1*A*, Cdc42 F28L facilitates the generation of RNA splicing products as compared with vector alone, and this effect is greatly enhanced when the RNA probe contains a specific m^{7}GpppG cap.

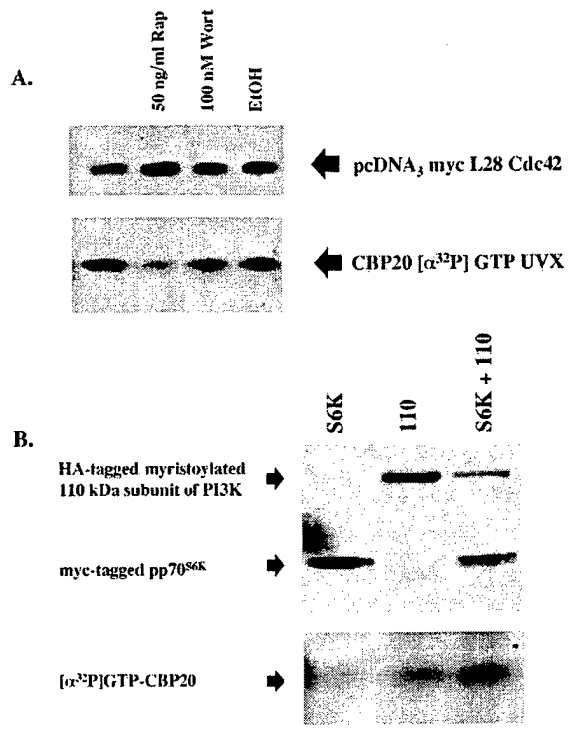

FIG. 2. **CBC activity promoted by Cdc42 is rapamycin-sensitive and can be promoted by PI3 kinase and S6K.** *A*, HeLa cells were transiently transfected with Myc-tagged Cdc42 F28L, serum-starved for 48 h, and then treated with rapamycin or wortmannin for 30 min. The cytosolic lysates were examined for the expression of Cdc42 using an antibody directed against the Myc-tag on the recombinant protein (*upper panel*). The nuclear lysates were assayed for CBC activation by photoaffinity labeling CBP20 with [α-^{32}P]GTP (*lower panel*). *B*, COS-7 cells were transiently transfected with wild type S6K (*S6K, lanes 1* and *3*) and/or a constitutively active, myristoylated 110-kDa catalytic subunit of PI3 kinase (*110, lanes 2* and *3*) and serum-starved for 48 h. The cytosolic lysates were assayed for the presence of S6K and the myristoylated 110-kDa subunit of PI3 kinase using antibodies directed against the Myc- and HA-tags on the recombinant proteins (*upper panel*). CBC activity in the nucleus was assayed by [α-^{32}P]GTP cross-linking to CBP20 (*lower panel*).

We have shown that the Cdc42 F28L mutant causes the transformation of NIH 3T3 cells (9), including the growth of these cells in low serum. Therefore, it is possible that Cdc42 F28L-mediated effects on splicing are not a direct outcome of a signaling pathway initiated by Cdc42 but rather reflect the fact that cells expressing Cdc42 F28L are able to grow in low serum, while the vector cell line shows little or no growth. To demonstrate that splicing is a direct outcome of Cdc42-coupled signaling events, we sought a Cdc42 mutant that would uncouple RNA splicing and cell growth. Splicing experiments were performed using lysates from NIH 3T3 cells that stably expressed either Cdc42 F28L or two effector loop mutants of Cdc42 in the activated background, Cdc42 F28L,C37A and Cdc42 F28L,Y40C (Fig. 2*B*, *lower right panel* compares the relative levels of expression of the Cdc42 proteins). In contrast to Cdc42 F28L, neither of the effector loop mutants will support the growth of cells in low serum (data not shown). Like Cdc42 F28L, the Cdc42 F28L,Y40C double mutant activates the splicing machinery, whereas Cdc42 F28L,C37A does not (Fig. 1*B*). Thus, the activation of splicing by Cdc42 is not dependent on cell growth. Furthermore, this mutational analysis suggests that the Cdc42 targets known to utilize position 40 in the effector loop, such as the CRIB (Cdc42/Rac-interactive binding) domain-containing proteins PAK, ACK, and WASP, are not involved in signaling to the splicing machinery. The identification of Cdc42 effectors for which position 37 is necessary has not been described to date, but it would appear that an effector of this class plays a pivotal role in the Cdc42-RNA processing pathway.

Given that the CBC is a downstream target for Cdc42, it seems likely that the regulation of CBC activity underlies the effects of Cdc42 on pre-mRNA splicing. To further explore how signals are conveyed from Cdc42 to the CBC, we tested the abilities of two small molecule inhibitors, rapamycin and wortmannin, to inhibit the activation of the CBC by Cdc42. These drugs inhibit FRAP and PI3 kinase, respectively. HeLa cells were transiently transfected with Cdc42 F28L, and 30 min prior to harvesting, either rapamycin or wortmannin was added. The cells were fractionated into nuclear and cytosolic components, and the nuclear fractions were assayed for CBC activity by measuring the photocatalyzed incorporation of [α-^{32}P]GTP into CBP20 as has been described previously (11). Rapamycin reduced CBC activity in the presence of Cdc42 F28L, whereas wortmannin did not (Fig. 2*A*, *upper panel* compares the relative amounts of Cdc42 F28L for the different cells assayed; the *lower panel* shows the relative incorporation of [α-^{32}P]GTP into CBP20 for the different conditions). This suggests that FRAP, but not PI3 kinase, is downstream of Cdc42 in the CBC pathway.

FRAP is thought to play a pivotal role in signaling pathways that regulate mRNA translation and is well documented as an upstream activator of the pp70 ribosomal S6 kinase (S6K) (18–21). This suggested that Cdc42 might signal to the CBC via a FRAP-S6K pathway. However, PI3 kinase is also an upstream activator of S6K (22), but wortmannin will not block the Cdc42 activation of the CBC. This would argue either that the Cdc42-FRAP pathway to the CBC is S6K independent or that involvement of PI3 kinase is either upstream of Cdc42 or is mediated through an independent pathway. To further assess the role of PI3 kinase and/or S6K in the activation of the CBC, COS-7 cells were transiently transfected with wild type S6K, a constitutively active form of PI3 kinase, the myristoylated 110-kDa subunit, or the two in tandem. The transfected cells were serum-starved for 48 h and harvested, and then cytosolic and nuclear lysates were generated. As shown in Fig. 2*B*, wild type S6K did not show a detectable activation of the CBC. In contrast, constitutively active PI3 kinase will activate the CBC, and this activation is enhanced when S6K is coexpressed with PI3 kinase. Taken together, these data show that although PI3 kinase is not necessary for the Cdc42-mediated activation of the CBC, it can signal to the CBC, probably through its ability to stimulate S6 kinase activity. The ability of Cdc42 to stimulate S6 kinase activity, independent of PI3 kinase activation, thus represents the most likely pathway by which Cdc42 regulates the CBC.

Interestingly, the 80-kDa subunit of the CBC, CBP80, contains two potential S6K phosphorylation consensus sites within its N terminus in a region that coincides with the bipartite nuclear localization signal (designated NLS1 and NLS2, see Fig. 4*C*). Thus we examined the ability of S6K to phosphorylate CBP80 *in vitro*. S6K was immunoprecipitated from COS-7 cells that had been cotransfected with S6K and the myristoylated 110-kDa subunit of PI3 kinase and was then incubated with recombinant CBC in a phosphorylation assay for varying periods of time or substrate (CBC) concentration (Fig. 3, *A* and *B*). S6K phosphorylated CBP80 to a level similar to that observed when equimolar amounts of GST-S6 were substituted for the CBC as the substrate in the assay (data not shown). To determine whether the phosphorylation occurred within the NLS region, an NLS mutant of CBP80 was examined. This mutant, CBP80 K17A,R18A, has been shown previously to inhibit the nuclear localization of CBP80 and presum-

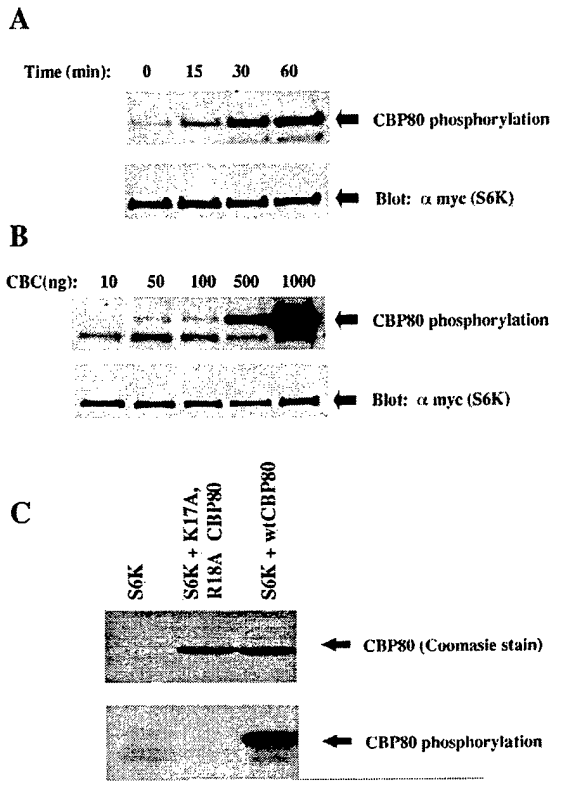

FIG. 3. **S6K phosphorylates CBP80 *in vitro* at a position within the N-terminal NLS.** *A*, S6K was immunoprecipitated from COS-7 cells which coexpressed S6K and the myristoylated 110-kDa subunit of PI3 kinase. CBC was added to the S6K in a phosphorylation reaction and incubated for increasing periods of time. CBP80 phosphorylation was detected by autoradiography (*top panel*), and S6K levels were detected by Western blotting against the Myc-tag on the recombinant S6K. *B*, immunoprecipitated Myc-S6K was incubated with increasing concentrations of CBC for 30 min in a phosphorylation reaction. The *top panel* represents CBP80 phosphorylation, and the *bottom panel* shows S6K levels in the assay. *C*, partially purified S6K was incubated with recombinant wild type CBC (*wt CBP80*) or an NLS mutant (K17A,R18A CBP80) in a phosphorylation reaction for 30 min. CBP80 protein levels were detected by Coomassie Blue stain (*top panel*), and phosphorylation of CBP80 was visualized by autoradiography (*bottom panel*).

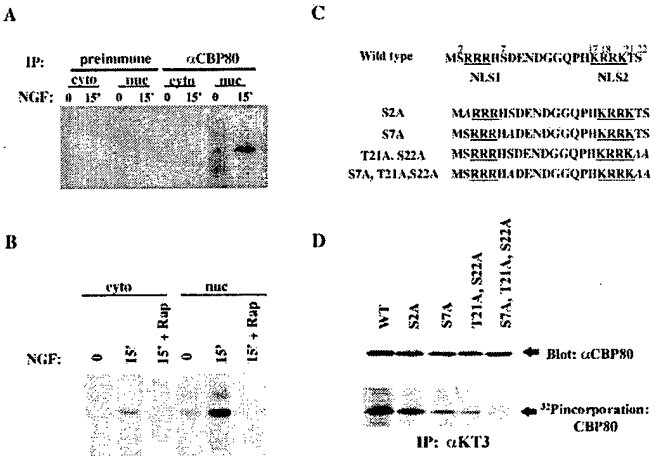

FIG. 4. **CBP80 undergoes a growth factor-responsive and rapamycin-sensitive phosphorylation within the N-terminal NLS *in vivo*.** *A*, PC12 cells were metabolically labeled with [^{32}P]orthophosphoric acid for 3 h and then stimulated with NGF (100 ng/ml) for 15 min. The cells were harvested and fractionated into cytosolic and nuclear lysates, and these lysates were then immunoprecipitated using either a specific CBP80 antiserum or a nonspecific preimmune rabbit serum. Immunoprecipitated proteins were separated by SDS-PAGE, and phosphorylated proteins were visualized by autoradiography. *B*, PC12 cells were metabolically labeled for 3 h with [^{32}P]orthophosphoric acid. Thirty minutes prior to NGF treatment (100 ng/ml, 15 min), 50 ng/ml rapamycin (*Rap*) was added as indicated. The cells were harvested and fractionated, and the cytosolic and nuclear lysates were immunoprecipitated using a specific CBP80 antiserum. Immunoprecipitated proteins were detected by autoradiography. *C*, N-terminal CBP80 sequence. The bipartite NLS is *underlined* and labeled as NLS1 or NLS2, and alanine mutations were made as shown. *D*, wild type and mutant CBP80 constructs were cloned into pcDNA$_3$ with a C-terminal KT$_3$ tag. The constructs were then transfected into HeLa cells, and the proteins were expressed for 24 h. The cells were metabolically labeled with [^{32}P]orthophosphoric acid for 3 h and treated with heregulin for 15 min. Lysates were prepared, and the transfected CBP80 proteins were isolated using an α-KT$_3$ antibody. The immunoprecipitated proteins were separated by SDS-PAGE, and CBP80 was detected by Western blotting using a specific CBP80 antibody (*top panel*) and by autoradiography (*bottom panel*).

ably importin binding to the CBC (23) and would no longer contain the S6K consensus site at NLS2. Indeed, this mutant fails to serve as a substrate for S6K phosphorylation (Fig. 3*C*).

Thus we are presented with an interesting possibility for how signaling from Cdc42 to the CBC proceeds, via a direct S6K-catalyzed phosphorylation of CBP80. We therefore set out to establish whether CBP80 phosphorylation is a viable signaling mechanism. Previously, we had shown that the CBC is strongly activated by NGF in PC12 cells and by heregulin in HeLa cells (11). Therefore, PC12 cells were metabolically labeled with [^{32}P]orthophosphoric acid in the presence or absence of NGF. The labeled cells were fractionated, and the CBC was immunoprecipitated from cytosolic and nuclear lysates using a CBP80 antiserum or preimmune serum. Fig. 4*A* shows that nuclear CBP80 is phosphorylated in a NGF-dependent manner in cells. Phosphorylation of CBP20 was not observed. Additionally, the NGF-dependent phosphorylation of CBP80 was blocked by a 30-min pretreatment of cells with rapamycin (Fig. 4*B*), emphasizing an involvement of FRAP and S6K in this RNA processing pathway.

N-terminal alanine mutant constructs of CBP80 were generated to confirm that the *in vivo* phosphorylation occurs within the S6K phosphorylation consensus sites (Fig. 4*C*). These CBP80 constructs were transiently transfected into HeLa cells that were then metabolically labeled with [^{32}P]orthophosphoric acid and then treated with heregulin to promote CBC activation. Following the labeling procedure, the mutant constructs were isolated from cell lysates using an antibody recognizing the KT$_3$ tag at the C terminus of the transfected proteins, and their phosphorylation states were assessed. The *in vivo* phosphorylation of CBP80 was reduced in the S7A and the T21A,S22A mutants and completely blocked in the CBP80 S7A,T21A,S22A mutant (Fig. 4*D*). All mutant constructs showed wild type nuclear localization as assessed by immunofluorescence (data not shown). Thus, the *in vivo* phosphorylation of CBP80 is consistent with a direct phosphorylation by S6K.

Finding that CBP80 is phosphorylated at two positions in cells was unexpected based on our *in vitro* phosphorylation experiments with S6K and the CBC where mutational analysis suggested a single phosphorylation within NLS2 of CBP80. We anticipated that the CBP80 K17A,R18A mutation would disrupt the S6K phosphorylation site at NLS2. However, it is possible that this mutation causes a larger structural perturbation such that the phosphorylation site within NLS1 has been lost as well. If this is the case, it is possible that S6K phosphorylates CBP80 at both positions in cells. Alternatively, phosphorylation of CBP80 by S6K may be restricted to the NLS2 site, and another, yet to be identified kinase is responsible for the phosphorylation at NLS1.

It is not clear whether the phosphorylation of CBP80 is occurring within the nucleus or cytoplasm of cells. Although the CBC is nuclear in the steady state, immunoelectrographic

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studies have suggested that the CBC accompanies the RNA particle through the nuclear pore (24), and it is generally thought that the CBC releases its cargo in the cytosol to the cytosolic cap-binding protein eIF-4E before rapidly shuttling back to the nucleus. Although mainly cytosolic, the S6K has also been reported in the nucleus, both as a longer isoform containing a NLS sequence (25–27) and as a kinase capable of the nuclear phosphorylation of the transcription factor, CREMτ (28). Given that the phosphorylation is occurring within the NLS of CBP80, it might be anticipated that a cytosolic phosphorylation would block importin binding to the CBC and thus nuclear localization. Such has been found to be true for diacylglycerol kinase ζ (29). However, we have not found phosphorylation of CBP80 by S6K to block an interaction with the importins *in vitro* nor have we observed that aspartic acid mutants, designed to mimic a phosphorylated CBP80, lose their nuclear localization.[2] Thus, at present both cytosolic and nuclear phosphorylations of CBP80 by S6K remain a viable mechanism.

S6K has been previously identified as an effector for Cdc42 (30) although this is the first report to suggest a functional consequence of this pathway, RNA splicing. S6K and its upstream activator, FRAP, have been well studied for their role in translational signaling. Phosphorylation of ribosomal S6 by S6K has been reported to have a positive effect on translation, especially for those transcripts that contain a polypyrimidine tract within their 5′-untranslated region (31). FRAP, in addition to activating S6K, also participates in the growth factor-regulated activation of the translation initiation complex, eIF-4F. Specifically, FRAP is thought to phosphorylate 4E-BP, a negative regulator of eIF-4E cap-binding activity (32–34). The phosphorylation of 4E-BP causes it to disengage from eIF-4E, which in turn promotes the association between eIF-4E and eIF-4G and the subsequent binding of capped RNA (35, 36). It is interesting that we have identified a potentially new role for these translational signaling players in CBC activation and RNA processing. This observation suggests a necessary coordination among aspects of gene expression that require the m^7G cap structure: cap-dependent RNA splicing, export, and translation. Given that there exists only two known cap-binding proteins, eIF-4E and the CBC, and that they process the same substrate in a manner that is both spatially and temporally distinct, common signaling elements would represent an elegant way to achieve a precise coordination of capped transcript processing.

The work reported here also raises some new possibilities regarding how Cdc42 controls cell growth. Cdc42 has been described to regulate aspects of cytoskeletal arrangement, vesicular trafficking, and transcriptional activation, and it has been demonstrated that at least one of these activities, vesicular trafficking through coatomer interactions, is necessary for Cdc42-induced transformation (6). It appears, however, that Cdc42 utilizes additional cellular targets when inducing malignant transformation. This is evidenced by the fact that distinct Cdc42 mutations will independently block cellular transformation including mutations in the effector loop at positions 37 and 40, as well as deletion of the insert region (10). These transformation targets have not been identified. However, activated Cdc42 proteins that are mutated at position 37 still bind CRIB (for Cdc42/Rac interactive binding) domain-containing target/effectors including the PAKs (p21-activated kinases), as well as the γ-coatomer subunit, which is the only Cdc42 target that has been directly implicated in Cdc42-mediated malignant transformation (6). Cdc42 proteins mutated at position 37 show a reduced affinity for the putative Cdc42/Rac target, IQGAP, but other mutational analyses have argued against a role for IQGAP in Cdc42-mediated transformation.[3] Thus, the fact that the Cdc42 F28L,C37A double mutant is both transformation-defective and unable to stimulate RNA splicing suggests an interesting connection between the ability of Cdc42 to mediate RNA processing and induce malignant transformation. In yeast, CBC knockouts demonstrate a poor growth phenotype (37), and dominant-negative CBC mutants are likely to have a negative impact on mammalian cell growth. Thus, we are currently examining the possibility that CBC mutants, such as phosphorylation-defective CBC, will block Cdc42-induced transformation. Such data would underscore both the importance of Cdc42 in coordinating diverse cellular functions including RNA splicing and the role of CBC-mediated RNA processing in regulated cell growth.

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Review

Signal Transduction and Post-Transcriptional Gene Expression

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Traditionally, growth factor-coupled signaling to the nucleus has been thought to be primarily directed toward transcriptional regulation. However, there are now increasing indications from a diversity of experimental systems that other aspects of RNA processing, including translation, lifetime and stability, and splicing are under strict growth factor control. In this review, we present the emerging evidence for growth factor signaling pathways that impact on these different RNA processing events. Particularly noteworthy is the realization that growth factor signaling through Ras can effect the regulation of two RNA cap-binding proteins, the cytosolic eIF-4E complex, which is necessary for initiating translation, and the nuclear cap-binding complex, the CBC, which plays a role in cap-dependent pre-mRNA splicing, U snRNA export and 3′-end processing. This, taken together with other findings that demonstrate the ability of stress response pathways and the small G protein, Cdc42, to activate the CBC, raises some interesting possibilities regarding how signaling to the two cellular RNA cap-binding protein complexes may coordinate the growth-coupled regulation of gene expression at the level of RNA processing.

Key words: eIF-4E / Nuclear cap-binding complex / RNA stability / Splicing / Translation.

Introduction

A strict regulation of gene expression is essential in maintaining proper cell function. Environmental cues, often in the form of extracellular growth factors, communicate with the cellular signal transduction machinery to influence a desired outcome such as cell growth, division or differentiation by ultimately altering the pattern of genes expressed within the cell. Indeed, a major area of research has entailed the precise mapping of signal transduction pathways that lead to the nucleus and influence the transcription of genes from DNA into RNA. One example of such a pathway utilized the Ras GTPase. The activation of a receptor tyrosine kinase by its cognate ligand recruits the Ras protein to the membrane via an assembly of adapter proteins, such as Grb2, and the guanine nucleotide exchange factor SOS, to yield an active, GTP-bound, Ras species. Ras then initiates the MAP kinase cascade, generating a signal that can ultimately communicate with a variety of different transcription factors, thereby affecting gene expression and cell fate. If such signals are not faithfully executed, the up- or down-regulation of certain genes regardless of context can result, and clinical manifestations such as cancer may arise. This underscores the important link between signal transduction and gene regulation. When attempting to understand how signals affect gene expression, it is important to consider other levels at which genes might be regulated in addition to transcription. Thus, this review focuses on emerging areas of signaling and gene expression including translation, RNA stability and pre-messenger RNA splicing, with the aim of highlighting what are likely to be exciting avenues for future research.

Signaling and Translational Control

Over the last decade, a large body of evidence has been gathered to describe what is commonly termed now as the translational signaling pathways, and these pathways have been the subject of many recent reviews (Brown and Schreiber, 1996; Peterson and Schreiber, 1998; Sonenberg and Gingras, 1998; Dufner and Thomas, 1999). Central to the connection between signaling and translational control is the cytosolic cap-binding protein, eIF-4E. eIF-4E is the component of the multisubunit translation initiation factor eIF-4F which is subject to regulation by multiple growth factors, cell stress and cell cycle arrest (Rhoads, 1993), and has been classified as a proto-oncogene as its overexpression causes cells to grow rapidly, lose contact inhibition, grow in soft agar, and form tumors in nude mice (De Benedetti and Rhoads, 1990; Lazaris-Karatzas *et al.*, 1990). Recruitment of eIF-4E to the ribosome occurs downstream of a phosphorylation of eIF-4E, and the phosphorylated form of the protein has an enhanced affinity for cap structures *in vitro* (Minich *et al.*, 1994). Thus, phosphorylation may serve as a molecular switching mechanism which can influence the ability of eIF-4E to recognize and bind capped RNAs, and in doing so, initiate translation.

The regulation of the phosphorylation and activation of eIF-4E is complex and appears to occur downstream of multiple signal transduction pathways (see Figure 1). Ras has been shown to function upstream of eIF-4E, as a dominant negative Ras T17N mutant can abrogate the NGF stimulated phosphorylation of eIF-4E in PC12 cells (Frederickson *et al.*, 1992), and can inhibit the mitogenic properties of overexpressed eIF-4E in NIH 3T3 cells (Lazaris-Karatzas *et al.*, 1990). Through the use of different MAP kinase inhibitors and different cell lines, the ERK, JNK/SAPK and p38 MAP kinase pathways have all been shown to have an effect on eIF-4E phosphorylation (Morley and McKendrick, 1997; Wang *et al.*, 1998). Thus, distinct MAP kinase pathways, acting either alone or in concert, may lead to eIF-4E activation under different cellular conditions. The identification of the kinase that is responsible for the *in vivo* phosphorylation of eIF-4E has not been forthcoming. PKC can phosphorylate eIF-4E *in vitro* (Tuazon *et al.*, 1990), whereas MAP kinase (Morley, 1996), p38 (Morley and McKendrick, 1997), and the MAP kinase substrates MAPKAPK-1 and MAPKAPK-2 cannot (Flynn and Proud, 1996). Recently, another kinase which can phosphorylate eIF-4E *in vitro* has been identified, Mnk1 (Fukunaga and Hunter, 1997; Waskiewicz *et al.*, 1997). Mnk1 is phosphorylated and activated by both Erk and p38, and its activation *in vivo* parallels that of eIF-4E (Wang *et al.*, 1998).

In addition to being positively regulated by phosphorylation, eIF-4E is also negatively regulated by a class of inhibitory proteins called eIF-4E binding proteins (4E-BPs) (Pause *et al.*, 1994). Three such binding proteins have been identified as 4E-BP1, 4E-BP2 (Lin *et al.*, 1994; Pause *et al.*, 1994), and 4E-BP3 (Poulin *et al.*, 1998). The 4E-BPs associate with eIF-4E in their nonphosphorylated state and competitively inhibit the association of eIF-4E with eIF-4G. Association of eIF-4G with eIF-4E dramatically enhances the ability of eIF-4E to bind cap structures (Haghighat and Sonenberg, 1997), therefore the complex between 4E-BP and eIF-4E inhibits cap binding. Upon phosphorylation, the 4E-BPs lose their affinity for eIF-4E and dissociate (reviewed in Sonenberg, 1996). Like eIF-4E, the phosphorylation of 4E-BP is subject to extracellular regulation by factors such as insulin (Brunn *et al.*, 1997b), although the signaling pathways that culminate in the phosphorylation of 4E-BP appear to be distinct from those resulting in the phosphorylation of eIF-4E (Morley and McKendrick, 1997). Rapamycin, the small molecule inhibitor of FRAP (Kunz *et al.*, 1993; Brown *et al.*, 1994; Sabatini *et al.*, 1994), potently inhibits 4E-BP phosphorylation (Brunn *et al.*, 1997b; Hara *et al.*, 1997; Morley and McKendrick, 1997; Gingras *et al.*, 1998) suggesting that 4E-BP phosphorylation is downstream of a novel FRAP signaling pathway. While FRAP shares homology with the family of PI kinase-related kinases, recent evidence suggests that it might be functioning as a protein kinase capable both of autophosphorylation (Brown *et al.*, 1995; Hara *et al.*, 1997) and phosphorylation of 4E-BP1 *in vitro* (Brunn *et al.*, 1997a, b; Hara *et al.*, 1997). Transfection studies have demonstrated a phosphorylation of 4E-BP1 upon cotransfection with FRAP which suggests, together with the rapamycin sensitivity of 4E-BP1, that FRAP may directly target 4E-BP1 *in vivo* (Brunn *et al.*, 1997b). Thus, FRAP is now suspected to be a pivotal signaling protein in translational control.

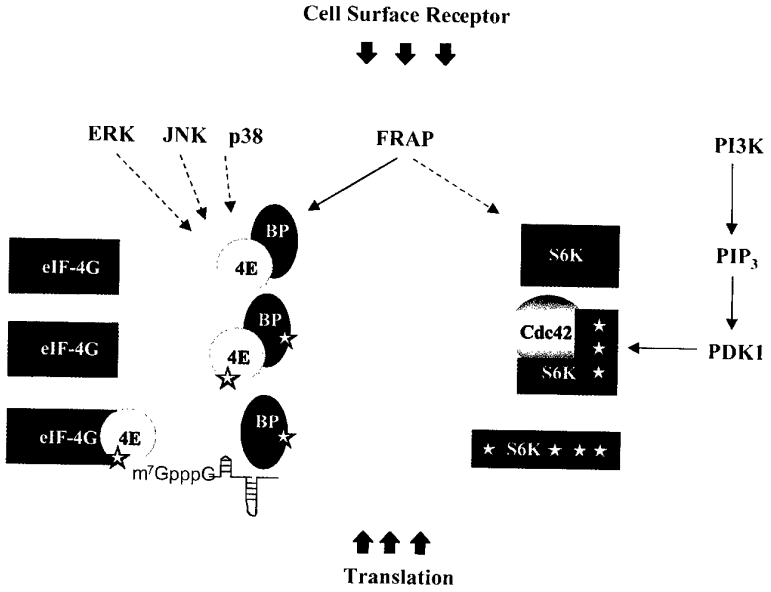

Fig. 1 Signaling Pathways Involved in Translational Control.
The left had panel depicts the activation of eIF-4E which enables it to bind capped RNAs. In its basal state, eIF-4E (4E) associates with 4E-BP1 (BP). In response to a stimulus, both eIF-4E and 4E-BP1 become phosphorylated (★), 4E-BP1 dissociates from eIF-4E,and the binding of capped mRNA to eIF-4E is enhanced both by phosphorylation and by its association with eIF-4G. The activation of S6 kinase (S6K) is shown in the right hand panel. The sequential phosphorylation of both rapamycin-sensitive and wortmannin-sensitive sites on S6K is necessary for its activation. Solid lines indicate direct interactions while dashed lines indicate indirect or speculative interactions.

In addition to regulating 4E-BP, FRAP is also upstream from the pp70 S6 kinase (S6K), which is activated by a wide array of mitogenic stimuli as well as by G1 progression. The activation of S6K is known to result in the phosphorylation of the S6 protein of the 40S ribosomal subunit (reviewed in Chou and Blenis, 1995) and this phosphorylation correlates with increased protein synthesis, particularly the translation of mRNAs with polypyrimidine tracts within their 5′ untranslated region (Jefferies *et al.*, 1997). Thus, S6K is believed to play a role in regulated gene expression at the level of translation. Rapamycin treatment of cells results in a specific inhibition of S6K, placing S6K downstream of FRAP signaling. Although an intact kinase domain is required for S6K activation by FRAP *in vivo*, no association between FRAP and S6K has been observed, suggesting that FRAP may not directly phosphorylate S6K (Brown *et al.*, 1995). The Rho-family GTPases Cdc42 and Rac have also been suggested to play a role in the activation of S6K (Chou and Blenis, 1996), and the PI3K target, PDK1 (3-phosphoinositide-dependent protein kinase 1), has recently been identified as the S6K-activating kinase (Alessi *et al.*, 1997; Pullen *et al.*, 1998). Thus, like eIF-4E, multiple signaling pathways appear to converge on S6K.

The number and diversity of signaling components involved in translational control is quite interesting and points to a necessity for strict regulation at this level of gene expression. Different MAP kinase pathways are also known to input into both transcriptional and other post-transcriptional levels of gene expression (see below) suggesting a coordination among all aspects of gene expression in the production of a given gene product. A role for FRAP and S6K outside of translational control is less defined at present. However, if a coordination of signaling events as they relate to different levels of gene expression is to evolve as a general mechanism in the regulation of genes, additional roles for FRAP and S6K can be anticipated. Also noteworthy is the role that the cap structure and cap-binding protein, eIF-4E, serve as endpoints for signals regulating translation initiation. Given that the eukaryotic cap structure also plays a role in RNA stability, splicing, 3′-end processing and transport, another emerging theme in gene expression may be the role of cap-binding proteins as signal integrators and executioners in post-transcriptional gene regulation (see below).

Signaling and RNA Stability

It has been well established that extracellular factors can influence the stability of different mRNAs (Jinno *et al.*, 1998; Watson, 1988; Lindstein *et al.*, 1989), including RNAs coding for c-myc, c-fos, colony-stimulating factor, tumor necrosis factor-α (Shaw and Kamen, 1986; Brawerman, 1987, 1989; Cleveland, 1988; Ross, 1988; Hargrove and Schmidt, 1989; Peltz *et al.*, 1991), and the interleukins (see below). Regulation at this level consists of both *cis*-elements within the 3′ untranslated region (UTR) of the mRNA (reviewed in Chen and Shyu, 1995), and *trans*-elements that recognize the *cis*-elements. Characteristic of *cis*-elements regulating RNA stability are tandem nucleotide repeats consisting of an AUUUA-type consensus (Malter, 1989; Bohjanen *et al.*, 1991; Brewer, 1991; Shyu *et al.*, 1991; Chen *et al.*, 1994; Chen and Shyu, 1994), and hence these sequences have been termed (A+U)-rich elements (AREs). Different protein factors that can recognize AREs have been observed (Malter, 1989; Bohjanen *et al.*, 1991; Brewer, 1991; Vakalopoulou *et al.*, 1991; Myer *et al.*, 1992; Port *et al.*, 1992; Levine *et al.*, 1993; Rajagopalan and Malter, 1994). One example, hnRNP A1, has been shown to vary in its affinity for the ARE in response to cellular localization and phosphorylation (Hamilton *et al.*, 1997), and PKC ζ has been suggested to function as an hnRNP A1 kinase (Municio *et al.*, 1995).

Recent studies have demonstrated that MAP kinase pathways play a crucial role in gene expression during the cytokine response by regulating, at least in part, the stability of particular RNAs. In human fibroblast-like synoviocytes, the expression of interleukin-6 (IL-6) in response to IL-1β can be inhibited by the p38 MAP kinase inhibitor SB203580, and this was shown to be due entirely to an effect on RNA stability (Miyazawa *et al.*, 1998). The cyclooxygenase-2 (COX-2) gene is similarly affected by SB203580 in human monocytes treated with bacterial lipopolysaccharide, although in this case the p38 MAP kinase pathway affects transcription as well as RNA stability (Dean *et al.*, 1999). IL-8 production is increased in response to MEKK1 in HEK-293 cells (Holtmann *et al.*, 1999), and three separate signaling pathways, the JNK/SAPK, NK-κB and p38 pathways, influence this response. While the JNK/SAPK and NK-κB pathways promote IL-8 transcription, the p38 pathway leads to an increased stability of the IL-8 message. An AU-rich region in the 3′ UTR of the IL-8 transcript is sufficient for the regulation of RNA turnover (Winzen *et al.*, 1999), suggesting that the increase in IL-8 message stability in response to the p38 pathway utilizes an ARE-dependent mechanism. The trans-mediator has not been identified, but MAP kinase-activated kinase 2 (MAPKAPK2), a downstream target of p38, will also induce IL-8 stability. While the above examples demonstrate the ability of p38 pathways to regulate RNA stability, RNA turnover has been shown to occur downstream of other pathways as well. For example, JNK/SAPK pathways have been implicated in the stabilization of IL-2 and IL-3 transcripts (Chen *et al.*, 1998), and the activation of the ERK pathway influences the stability of the message coding for the muscarinic receptor (Lee and Malek, 1998).

Another link between signal transduction and RNA stability has come with the identification of the Ras GAP-SH3 binding protein (G3BP) as a 3′ UTR endonuclease (Gallouzi *et al.*, 1998). G3BP is a 52 kDa protein containing an RRM (RNA recognition motif) domain type RNA-binding motif. G3BP interacts with Ras GAP in membrane fractions obtained from serum stimulated cells but this interaction is not detected in quiescent cells. Additionally, in quiescent cells, G3BP is hyperphosphorylated on serine

residues and this phosphorylation is necessary for full activation of the endonuclease activity. It is not yet clear whether G3BP RNase activity is directed toward specific transcripts, or if it is a more general sensor of AU-containing RNAs. If the latter were true, the activation of G3BP upon serum withdraw-al could ensure the rapid degradation of proliferative mRNAs.

Signaling and Pre-mRNA Splicing

The differential splicing of pre-mRNAs is a major post-transcriptional control point in the regulation of many genes. Many higher eukaryotic genes contain multiple introns allowing for the expression of different isoforms in a developmental or tissue specific manner. The regulation of splicing is dictated by both *cis*-elements within the pre-mRNA, and *trans*-elements in the form of splicing enhancers or silencers (reviewed in Moore *et al.*, 1993; Adams *et al.*, 1996; Manley and Tacke, 1996; Wang and Manley, 1997). While it seems obvious that extracellular factors might play an important role in constitutive and alternative splicing by regulating the expression and/or activity of *trans*-activating factors, little is known about how signal transduction pathways input into the splicing machinery. Therefore, for the purpose of this discussion, we will focus on the signal transduction aspect of splicing.

It has been demonstrated that extracellular factors can stimulate the splicing machinery. In particular, it has been shown that heregulin (a growth factor that stimulates Neu/ErbB2 tyrosine kinase activity through the formation of Neu/ErbB2-ErbB3 heterodimers) increases the ability of HeLa cell extracts to support splicing *in vitro* (Wilson *et al.*, 1999). This data suggests that one or more factors must be activated downstream of receptor-ligand interactions in order for splicing to commence. Growth factors have also been shown to induce alternative splicing *in vivo*. Some examples of this include the alternative splicing of Trk pre-mRNA in response to NGF in PC12 cells (Barker *et al.*, 1993), and the alternative splicing of phosphotyrosine phosphatase PTB-1B in EGF treated HeLa cells (Shifrin and Neel, 1993).

A dominant-negative Ras mutant (Ras T17N) blocks NGF-induced increase in the levels of Agrin mRNA and alternative splicing in PC12 cells (Smith *et al.*, 1997). Both Ras and PKC have also been implicated in the alternative splicing of CD44 in T cells (Konig *et al.*, 1998). The RNA sequences necessary for exon inclusion include two splice silencer regions and an exon recognition element within the v5 exon of the CD44 gene. The exon recognition motif is purine rich, suggesting that SR proteins might bind to this region and aid in exon recognition (see below). It is not clear whether phorbol esters are signaling to the silencer regions, the exon recognition motif, or both, but transacting factors interacting with these regions are good candidates for downstream effectors of the PKC and/or Ras signaling pathways. Ras and PKC have also been implicated in the alternative splicing of the protein tyrosine phosphatase, CD45 in T cells (Lynch and Weiss, 2000). Recently, a novel downstream target of the Ras/Map kinase pathway has been identified in *Drosophila*. Sequence analysis suggests that this gene, *split ends*, is a member of the RRM family of RNA-binding proteins (Rebay *et al.*, 2000). It will be of interest to determine whether this gene encodes a protein that is indeed capable of RNA-binding, and if so, if this protein might be capable of mediating Ras effects on alternative splicing.

There has also been some evidence to suggest that Src plays a role in RNA processing at the levels of RNA splicing and transport (Neel *et al.*, 1995). An intact kinase domain is required for these activities (Gondran and Dautry, 1999), whereas the SH3 domain of Src is not necessary. Given that the SH3 domain is known to interact with the RNA-binding protein targets, Sam68 (Fumagalli *et al.*, 1994; Taylor and Shalloway, 1994) and hnRNP K (Weng *et al.*, 1994) (see below), it would appear that neither of these RNA-binding proteins are involved in the Src-mediated effects on splicing or transport.

RNA-Binding Proteins as Mediators of Extracellular Signals

From the examples mentioned above it seems clear that extracellular signals are being propagated through the cell to affect RNA processing. In the case of translational control, the mechanisms by which signaling pathways are affecting translation initiation have been elucidated in some detail. However, for RNA stability and pre-mRNA splicing, the way in which signals are ultimately exerting their effects on the RNA is less clear. There have been some reports of RNA-binding proteins that are linked to signal transduction and/or the cell cycle, making such proteins attractive candidates as signaling endpoints involved in post-transcriptional gene expression. The remaining discussion will briefly describe a few of the proteins and their connections with RNA processing and signal transduction.

The SR proteins are a group of RNA-binding proteins containing one or two RNP-type RNA-binding domains as well as arginine-serine dipeptide repeats (RS domains). SR proteins appear to be important in both protein-RNA and protein-protein interactions, and can have both positive and negative effects on splicing (Fu, 1995; Manley and Tacke, 1996; Wang and Manley, 1997). Phosphorylation of serine residues within the RS domain has been observed and suggested to play a role in pre-mRNA splicing (Mermoud *et al.*, 1994), and kinases which can phosphorylate SR proteins have been identified. Two of these kinases are SR protein kinase 1 (SRPK1) (Gui *et al.*, 1994a, b), and Cdc-2-like kinase/serine-threonine-tyrosine kinase (Clk/Sty) (Colwill *et al.*, 1996). The kinase activity of SRPK1 is highest in mitosis suggesting a connection with the cell cycle (Gui *et al.*, 1994a). Whether either of these kinases, or an unidentified SR protein kinase, is an endpoint for signal transduction pathways remains to be determined.

Heterogeneous ribonucleoprotein K (hnRNP K) is an hnRNP with multiple links to both signal transduction and gene expression. Known binding partners for hnRNP K include DNA, RNA, and a host of transcription factors, the proto-oncogene Vav, Src family members, and PKC (Matunis *et al.*, 1992, 1994; Dejgaard *et al.*, 1994; Maa *et al.*, 1994; Ostrowski *et al.*, 1994; Weng *et al.*, 1994; Bustelo *et al.*, 1995; Taylor *et al.*, 1995; Van Seuningen *et al.*, 1995a, b; Denisenko *et al.*, 1996; Michelotti *et al.*, 1996; Denisenko and Bomsztyk, 1997). The interaction between hnRNP K and PKC can be inhibited by RNA (Schullery *et al.*, 1999), and hnRNP K-RNA interactions can likewise be disrupted by the tyrosine phosphorylation of hnRNP K (Ostrowski *et al.*, 2000). Thus it has been suggested that hnRNP K may serve as a scaffolding protein which can effectively couple signaling proteins and proteins involved in gene expression.

A related protein to hnRNP K, Sam68, is the major Src-associated, tyrosine-phosphorylated protein in mitotic cells (Fumagalli *et al.*, 1994; Taylor and Shalloway, 1994). Sam68 has been shown to bind to RNA through its hnRNP K homology (KH) domain, and tyrosine phosphorylation can disrupt this binding (Taylor and Shalloway, 1994; Wang *et al.*, 1995). In addition to binding Src and other Src family members (Fusaki *et al.*, 1997), Sam68 has been shown associate with numerous signaling molecules including Grb2, PLCγ1, p85, Cbl, Jak3 and Ras-GAP (Neet and Hunter, 1995; Richard *et al.*, 1995; Fusaki *et al.*, 1997; Guitard *et al.*, 1998). Given that Sam68 shows a preference for binding to RNAs containing poly (A) and poly (U) in an *in vitro* selection assay (Lin *et al.*, 1997), it is possible that Sam68 might target AU-rich regions mediating RNA stability *in vivo*.

Recently, the nuclear cap-binding complex (CBC) has been shown to be a signal transduction target (Wilson *et al.*, 1999). The CBC binds to the ^{7}methylguanosine cap-structure of RNAs transcribed by RNA polymerase II (Izaurralde *et al.*, 1994, 1995; Kataoka *et al.*, 1994, 1995), the same substrate to which the translation initiation factor eIF-4E binds. In binding the cap, the CBC has been shown to enhance the splicing of pre-mRNAs (Ohno *et al.*, 1987; Izaurralde *et al.*, 1994; Lewis *et al.*, 1996a, b) and the nuclear export of U snRNAs (Izaurralde *et al.*, 1995), as well as facilitate 3′ end-processing (Flaherty *et al.*, 1997). The CBC is stimulated to bind cap structures in response to various growth factors, cell stress, and cell cycle arrest, as well as in response to activated forms of Ras and Cdc42 (Wilson *et al.*, 1999). This pattern of cap binding is similar to that observed for eIF-4E, suggesting that common signal transduction pathways between these two cap-binding proteins may exist. This is especially attractive given the possibility that a coordination of cap binding between the nuclear and cytosolic proteins might be necessary to ensure the temporal processing of capped RNAs.

Unlike eIF-4E, the detailed events that culminate in the activation of the CBC, such that it is able to bind capped RNAs, have yet to be reported. This information should be of significant value, particularly if signaling to the CBC is demonstrated to regulate one or more of the aspects of RNA processing. Given the similarities between those stimuli that activate eIF-4E and the CBC, it is attractive to envision that the CBC might be regulated in a manner that is mechanistically similar to eIF-4E. That is, multiple signaling pathways may lead to the activation of the CBC, as is suggested by the fact that both the Ras and Cdc42 proteins can simulate CBC activity (Wilson *et al.*, 1999). Cdc42 has numerous downstream effectors, one of which is S6K (Chou and Blenis, 1996). Perhaps rapamycin-sensitive pathways can influence CBC activation as well as translational control, a suggestion which is made more plausible by the observation that the 80 kDa subunit of the CBC, CBP80, can be phosphorylated by S6K within its nuclear localization sequence (NLS) *in vitro*, and that the CBC can be activated by the transfection of S6K *in vivo* (Wilson, unpublished data). It will be of interest to see if the CBC, like eIF-4E, is phosphorylated in response to extracellular stimuli *in vivo*, and whether such a phosphorylation might contribute to the regulation of the CBC. Additionally, an examination of different Cdc42 effector mutants will prove valuable in dissecting out the Cdc42 pathway that is leading to the CBC and RNA processing.

CBC-binding proteins might also participate in the regulated cap binding by the CBC. Indeed, the importin heterodimer has been shown to interact with the CBC and affect cap binding (Gorlich *et al.*, 1996). Specifically, α-importin binds to the NLS region of CBP80, and when bound, the CBC is competent to bind capped RNA. β-importin

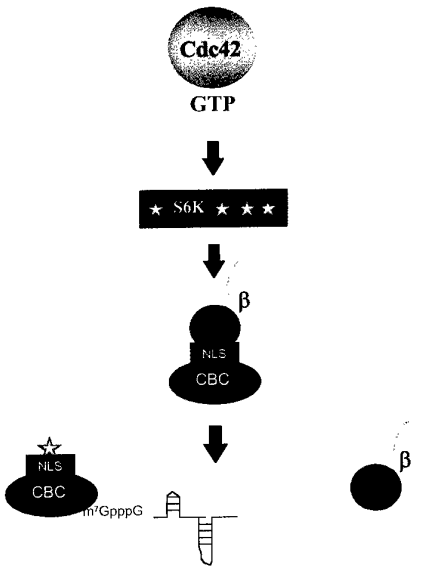

Fig. 2 Model for Regulated Capped RNA-Binding by the CBC. One possible pathway leading to the activation of the nuclear cap-binding complex (CBC) utilizes the small GTP-binding protein Cdc42, and its putative effector, S6 kinase (S6K). (α)- and (β)-importin have been shown to affect the ability of the CBC to bind capped RNAs and therefore may be involved in regulated RNA-binding by the CBC. A stimulus-driven phosphorylation within the nuclear localization sequence (NLS) of the CBC might disrupt importin binding and ensure cap-binding by the CBC.

can bind to the CBC-bound α-importin. The CBC within the context of this larger complex, however, cannot bind capped RNAs. It has been suggested that these protein-protein interactions might ensure CBC-capped RNA release in the cytoplasm, since β-importin is largely cytosolic (Gorlich *et al.*, 1996). Another scenario might suggest that the importins function as negative regulators of the CBC, analogous to the relationship between the 4E-BPs and eIF-4E. In response to a signal, the importins may dissociate from the CBC, allowing the CBC to bind capped RNAs. A phosphorylation event within the NLS could be sufficient to disrupt importin binding as has been demonstrated for other NLS-containing proteins such as diacylglycerol kinase-ζ (Topham *et al.*, 1998). In addition to negative regulators, it will be interesting to determine whether there exists CBC-binding proteins that enhance the affinity of the CBC for cap-structures. At the present time, we can draw a model of CBC activation that is similar to what has been proposed for the regulation of eIF-4E (Figure 2). Although differences between the activation of these two cap-binding proteins undoubtedly exist, such a model can serve as a starting point in the investigation of the regulation of the nuclear cap-binding protein. Likewise, it will of great interest to determine if CBC-coupled RNa-processing events are similarly regulated in response to extracellular stimuli, and if so, whether signals leading to the CBC are causal to this regulation.

Conclusion

While the connection between signal transduction and transcriptional control has been well established, the role of signaling in post-transcriptional gene expression has not, to date, been appreciated to the same degree. However, as outlined in the preceding sections, there is every reason to believe that the regulation of RNA processing at the levels of splicing, stability, and translation will be every bit as important as the regulation of transcription. Indeed, signaling to translational control has been demonstrated in some detail and appears to utilize multiple cellular signaling systems. Additionally, abnormalities in translational control, such as the overexpression of eIF-4E, can result in malignant transformation. How signal transduction feeds into other areas of post-transcriptional gene regulation, such as RNA stability and pre-mRNA splicing, is not well defined at present but suggestions as to how signals might influence these processes are beginning to emerge. The different MAP kinase pathways appear to influence all levels of post-transcriptional gene expression discussed herein. Identification of RNA processing endpoints for these pathways will be pivotal in elucidating how distinct pathways exert their effects in the expression of a given gene. While there are likely to be numerous targets that serve as trans-activators mediating the expression of specific genes, equally important will be the general factors that may play a more universal role in ensuring that genes are expressed only under appropriate cellular conditions. The cap-binding proteins, eIF-4E and the CBC, may function in this regard. As gene expression checkpoints, these proteins 'sense' the cellular environment by the multiple signals that culminate in their activation. In the absence of such signals, these cap-binding proteins will not bind to capped-RNA, thereby ensuring that 'leaky' messages, which might have otherwise had an aberrant effect on cell growth, are processed no further. Ultimately, for us to understand the totality of regulated gene expression, we will need to appreciate the regulation as it is occurring simultaneously on multiple levels. Thus the emerging field of signal transduction and post-transcriptional gene regulation promises to be as fruitful as it is complex.

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